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Detection of Activated Pepsin in Bronchoalveolar Lavage and Acute Cellular Rejection in Lung Transplant Recipients

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Abstract

Activated pepsin (pepA) in bronchoalveolar lavage (BAL) fluid may be a biomarker of gastric aspiration. We sought to *i*) evaluate the association of pepA in BAL fluid with acute cellular rejection (ACR) in a cohort of lung transplant recipients (LTRs), *ii)* assess the association between pepA and isolation of typical gastrointestinal microorganisms from BAL fluid, and *iii)* explore the accuracy of using pepA concentration as a biomarker of ACR. After IRB approval, we conducted a retrospective observational study analyzing posttransplant BAL fluid samples and concomitant transbronchial biopsies (TBBs) obtained from LTRs who underwent at least two routine surveillance bronchoscopies between March 2020 and August 2022. A total of 349 BAL samples and paired TBBs from 120 LTRs were analyzed. Thirty-five LTRs (29.2%) had at least one episode of ACR during the study period. Most recipients (83.3%) had detectable

pepA in at least one BAL sample. LTRs with detection of pepA any time after LTx had a higher likelihood of ACR (OR 9.79 [CI95: 1.26-79.26], P = 0.009). The pepA concentration trended higher as the histological grade of ACR increased, and a cut-off of >2.45 ng/mL provided a sensitivity of 63.3% and specificity of 57.3% to detect ACR. In conclusion, detectable pepA in BAL samples is common among LTRs and was associated with the occurrence of ACR. Furthermore, the BAL pepA concentration trended higher as the histological ACR grade increased; however, this biomarker has several drawbacks if used alone for the detection of ACR, and cautious interpretation is recommended.

Keywords
Activated pepsin; bronchoalveolar lavage; aspiration; lung transplant recipients; acute cellular rejection

1. Introduction

Lung transplantation (LTx) is life-saving for some patients with end-stage lung disease; however, long-term survival is shorter among lung transplant recipients (LTRs) than other solid organ transplant recipients. Acute cellular rejection (ACR), a T cell immune response against antigens related to the donor's major histocompatibility complex [1], is a significant source of post-LTx morbidity and mortality and also a major risk factor for chronic lung allograft dysfunction (CLAD), the most common cause of death among long-term LTx survivors [2, 3].

The incidence of ACR among LTRs is high, with 28% of recipients experiencing at least 1 episode within the first year after LTx [4]. The clinical presentation of ACR is variable and ranges from asymptomatic to severe hypoxemic respiratory failure. Hence, most transplant centers use surveillance bronchoscopies to screen asymptomatic recipients [2-4] and diagnose ACR based on histological findings of transbronchial biopsies (TBBs) [5].

In addition to immune-mediated mechanisms of lung injury, such as ACR and antibody-mediated rejection (AMR), non-alloimmune injury is also common and can be mediated via aspiration of gastric contents in LTRs with gastroesophageal reflux disease (GERD) [6-8]. Animal models utilizing orthotopic LTx have shown that GERD and the resultant aspiration augment inflammatory cellular infiltration (primarily allograft CD8+ T cells), production of proinflammatory cytokines, and production of profibrotic growth factors (e.g., TGF-beta) [9-12], indicating that aspiration may play a role in the development of AMR and ACR. Moreover, repetitive aspiration events in non-LTx mice models also lead to chronic inflammation characterized by the presence of macrophages and higher levels of TGF-beta, TNF-alpha, and other proinflammatory cytokines, suggesting that recurrent aspiration may be associated with a broad spectrum of pulmonary diseases such as asthma, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and bronchiectasis [13, 14].

Pepsin is a peptidase secreted as a zymogen called pepsinogen by chief cells of the gastric mucosa [15]. It is activated with exposure to low gastric pH (i.e., activated pepsin [pepA]). Thus, isolation of this endopeptidase within the respiratory tract suggests gastric aspiration and may be an early biomarker of ACR [16-19]. With this study, we aimed to *i)* evaluate the association between pepA in bronchoalveolar lavage (BAL) fluid and ACR in a large cohort of LTRs, *ii*) assess the association

between detectable pepA in BAL fluid and isolation of typical gastrointestinal (GI) microorganisms from the BAL fluid samples, and *iii*) explore the accuracy of using pepA BAL fluid concentration as a biomarker of ACR and propose an optimal cut-off concentration for this purpose.

2. Materials and Methods

2.1 Study Design and Setting

This was a single-center, retrospective observational study that analyzed BAL fluid samples and concomitant TBBs from LTRs transplanted between June 2019 and May 2022 at St. Joseph Hospital and Medical Center, Phoenix, AZ, USA; this study also included an accuracy test assessment for pepA as a potential biomarker for the detection of ACR. Patient data were abstracted from the electronic medical records, and de-identified data were stored and managed using the Research Electronic Data Capture (REDCap) system.

The Institutional Review Board of St. Joseph's Hospital and Medical Center approved the collection and publication of the data under the Norton Thoracic Institute Foregut Umbrella Protocol (PHXU-21-500-136-73-18, Date: 30/09/2022). Written patient consent was waived due to the study design, and good practice guidelines were followed according to the Helsinki Declaration. The STROBE statement and checklist were used as a guide to determine the contents and ensure the quality of the manuscript (Supplementary material Table S1).

2.2 Study Population

We included BAL fluid samples as well as the concomitant TBB pathology reports obtained from single or bilateral LTRs with documentation of at least two bronchoscopies performed during routine surveillance bronchoscopy or when otherwise indicated by the transplant pulmonologist within the study period. The complete study inclusion and exclusion criteria are presented in Supplementary material Table S2.

2.3 Bronchoalveolar Lavage Assessment

All LTRs undergo surveillance bronchoscopy with BAL, bacterial, fungal, and mycobacterial cultures, respiratory viral PCR, and TBB at 1, 3, 6, 9, and 12 months after LTx. Bronchoalveolar lavage is performed by instilling 60 ml of normal saline into the right middle lobe with subsequent aspiration of fluid. If graft rejection is suspected, a non-scheduled TBB is performed. If ACR is detected, the patient undergoes an additional follow-up one month after the treatment completion. All bronchoscopies included in this study were performed by the same pulmonologist (AA) to ensure technically and operatively homogenized data.

Biochemical evaluation of BAL and measurement of pepA concentration was performed by a single regional external laboratory (ARUP Laboratories, Salt Lake City, Utah). The specimen is received frozen and fixed in saline solution for the measurement of pH, total protein, and pepA concentration. For this study, pepA was measured using a semi-quantitative enzymatic assay. Importantly, the measurement of pepA is not yet standardized, and a consensus on diagnostic thresholds is lacking; however, the laboratory suggests the following reference values: <12.5 ng/mL negative, 12.5-100 ng/mL moderately positive, and >100 ng/mL strongly positive. Notably, the suggested ranges from this laboratory are based on a pediatric population (as indicated in the

reports) and unrelated to a correlation with ACR. Since a threshold for an association of pepA concentration in BAL fluid with ACR has not been established, we used a dichotomous variable (detectable [>0.1 ng/mL] or undetectable) to measure the strength of the association between pepA and ACR. The laboratory did not provide a reference range for pH or total protein concentrations in BAL fluid.

In addition to pepA, BAL fluid was cultured for bacteria, fungi, and mycobacteria, and viruses were identified by employing RT- and QT-PCR techniques using a standardized viral pathogen panel that included influenza, parainfluenza, adenovirus, rhinovirus, human metapneumovirus, and coronaviruses (229E, OC43, HL63, HKU1, SARS-CoV-2).

2.4 Evaluation of Transbronchial Biopsies

Tissue specimens obtained via TBB were preserved in formalin and sent to the pathology department where the tissue is processed and microscopically evaluated for ACR. Our institution uses The International Society for Heart and Lung Transplantation ACR grading as follows: A0, none; A1, minimal or focal; A2, mild; A3, moderate; A4, severe; AX, inadequate sample.

2.5 Data and Statistical Analysis

Descriptive statistics were applied, and all data were assessed for normality using the Shapiro-Wilk test. Continuous variables are reported as median and interquartile range; categorical variables are reported as count and proportion. To assess differences between categorical variables, the Chi-square test was used; whereas the Mann-Whitney U test or the Kruskal-Wallis test for continuous nonparametric data were applied as appropriate. To determine associations between detection of pepA and ACR, contingency tables were created, and the results were reported as odds ratios (OR). Furthermore, a receiver operating characteristic (ROC) curve was employed to assess the accuracy of using pepA concentration (i.e., index test) to identify ACR as determined by the pathological report of the paired TBB (i.e., reference standard); the optimal cut-off value for ACR detection using pepA BAL fluid concentration was determined using the Youden index. Potential correlations between covariates as well as confounders were identified using Pearson's r correlation analysis. A P value < 0.05 was considered statistically significant. SPSS Statistics v29.0 (IBM, SPSS Inc. Armonk, NY, USA) was used for the analysis.

3. Results

3.1 Cohort Characteristics

A total of 187 LTRs underwent BAL between May 2020 and June 2022; 44 did not have any subsequent pepA measurements, and 23 had only one pepA measurement without a concurrent biopsy. Thus, 120 recipients met the inclusion criteria; 63.3% were male, the median age was 65 years, and the median BMI was 25.8 kg/m^2. Most patients were categorized within UNOS Group D at the time of transplant (n = 84 [70%]), and all but one patient underwent a bilateral LTx (n = 119 [99.2%]). Before and after LTx, 64 (49.2%) and 50 (38.5%) patients, respectively, had evidence of pathological acid exposure (i.e., abnormal DeMeester score [≥14.73]). The only significant difference between LTRs who developed ACR at any point after LTx and those who did not was the

preoperative DeMeester score. Table 1 summarizes the demographic and clinical characteristics of the cohort as well as the pH monitoring parameters before and after LTx.

Table 1 Demographics, transplantation characteristics, and pH monitoring parameters.

Characteristics	Study Cohort (N = 120)	LTRs with ACR ≥A1 (n = 35)	LTRs without ACR (n = 85)	*P-value*
Demographics				
Age, years, median (IQR)	65 (58-71)	64 (57-70)	66 (59-71)	0.178
Male sex, no. (%)	76 (63.3)	24 (68.6)	52 (61.2)	0.445
BMI, kg/m^2, median (IQR)	25.8 (23.7-28.6)	25.7 (23.7-27.4)	25.8 (23.7-28.9)	0.462
Transplantation Type				
Bilateral lung transplant, no. (%)	119 (99.2)	35 (100)	84 (98.8)	0.634
UNOS Primary Diagnostic Group				
UNOS group A, no. (%)	25 (20.8)	6 (17.1)	19 (22.3)	0.626
UNOS group B, no. (%)	7 (5.8)	2 (5.7)	5 (5.9)	1
UNOS group C, no. (%)	4 (3.3)	2 (5.7)	2 (2.4)	0.351
UNOS group D, no. (%)	84 (70)	25 (71.4)	59 (69.4)	0.637
Pre-LTx pH Monitoring Parameters				
DeMeester score, median (IQR)	15.6 (4.9-33.1)	26.7 (6.5-42.3)	12.5 (4.1-28.2)	**0.02**
Total AET, %, median (IQR)	4.7 (0.8-9.3)	6.4 (1.4-12.2)	3.3 (0.6-8.4)	0.099
Post-LTx pH Monitoring Parameters				
DeMeester score, median (IQR)	9.6 (3.6-24.2)	10.2 (3.9-24.4)	9.4 (3.4-24.0)	0.63
Total acid exposure time, %, median (IQR)	2.6 (0.5-7.0)	2.5 (0.7-7.7)	2.7 (0.5-6.7)	0.961

Assessment of differences between LTRs who developed ACR and those who did not was conducted using the Mann-Whitney U test. Bold indicates statistically significant p-values ($p < 0.05$). Abbreviations: AET, acid exposure time; BMI, body mass index; IQR, interquartile range; UNOS, United Network for Organ Sharing; UNOS group (A), obstructive lung disease; (B), pulmonary vascular disease; (C), cystic fibrosis; and (D), restrictive lung disease.

3.2 PepA Concentration and ACR Grading

First, the biochemical characteristics and pepA concentrations of 349 BAL samples were described and compared according to the histological ACR grade reported for the paired TBB. The median time from LTx to TBB and BAL was 186.5 days (IQR, 91-304.5), and the median number of procedures per recipient was 3. The median BAL pepA concentration was 7.8 ng/mL (IQR, 2.25-13.9), the median total protein concentration was 0.25 ng/mL (IQR, 0.144-0.415), and the median

pH was 5.5 (IQR, 5.0-5.8). Pathology reported AX in 21 (6%) biopsies, ACR ≥A1 in 49 (14.1%) biopsies, and A0 in 279 (79.9%) biopsies. We observed a trend toward a higher pepA concentration as the histological ACR grade increased: the median pepA concentration was 1.5 ng/mL (IQR, 0-7.2) for grade A0 biopsies, 4.4 ng/mL (IQR, 0-6.9) for grade A1 biopsies, 3.5 ng/mL (IQR, 0.4-9.7) for grade A2 biopsies, and 10.75 ng/mL (IQR, 1.5-20) for grade A3 biopsies. Similarly, a trend toward higher total protein concentration in BAL samples as histological ACR grade increased was identified (Table 2).

Table 2 Pepsin A concentrations, biochemical characteristics of bronchoalveolar lavage fluid samples, among the different histological grades of acute cellular rejection.

Covariate	**TBB Reporting A0 Grade (n = 279)**	**TBB Reporting A1 Grade (n = 25)**	**TBB Reporting A2 Grade (n = 22)**	**TBB Reporting A3 Grade (n = 2)**	***P-value***
Pepsin A, ng/mL	1.5 (0-7.2)	4.4 (0-6.9)	3.5 (0.4-9.7)	10.75 (1.5-20)	0.163
BAL pH,	5.2 (5-5.5)	5.2 (5-5.5)	5.5 (5-5.8)	5.4 (5-5.8)	0.388
BAL protein concentration, ng/mL	0.2 (0.12-0.4)	0.23 (0.2-0.31)	0.34 (0.2-0.9)‡	0.55 (0.3-0.8)	**0.049**

All data presented as median (interquartile range). Assessment of differences between ACR grades was conducted using the Kruskal-Wallis test. Bold indicates statistically significant p-values ($p < 0.05$). ‡ p-value < 0.05 at 2-sided tests compared to the A0 grade of acute cellular rejection. Abbreviations: BAL, bronchoalveolar lavage; TBB, transbronchial biopsy.

Further, bivariate analyses were conducted to explore associations between the detection of pepA (≥0.1 ng/mL) at any point after LTx and ACR. Overall, a total of 35 (29.2%) LTRs had ACR (any grade) and 85 (70.8%) did not. Notably, almost all patients with ACR had detectable pepA in at least one BAL sample (97.1%), whereas the proportion of LTRs without ACR presenting detectable pepA was significantly lower (77.6%). LTRs with detectable pepA at any point after LTx had a higher likelihood of presenting with ACR (OR 9.79 [CI95 1.26-79.26], P = 0.009). Importantly, detection of any concentration (≥0.1 ng/mL) of pepA in BAL fluid at any point after LTx had a sensitivity of 97.1% (CI95: 85.1-99.9%) and specificity of 22.4% (CI95: 14.0-32.7%) for the identification of patients who presented with ACR after LTx. The positive predictive value was 34% (CI95: 31.2-36.9%), and the negative predictive value was 95% (CI95: 72.6-99.3%). Vice versa, when considering all TBBs documenting any grade of ACR (n = 49) and those without evidence of rejection (i.e., A0 grade, n = 279), we identified a slightly higher likelihood of ACR if pepA was detected in the concomitant BAL fluid (OR 1.94 [CI95 0.99-3.82], P = 0.0519; Table 3).

Table 3 Cross-tabulation exploring the association between detection of activated pepsin in bronchoalveolar lavage fluid and acute cellular rejection in lung transplant recipients.

Detection of pepA Among LTRs During the Study Period			
Covariate	**LTRs with ACR ≥A1 (n = 35)**	**LTRs without ACR (n = 85)**	**Row Total, no (%)**
Detectable pepA, no (%)	34 (97.1)	66 (77.6)	100 (83.3)
Undetectable pepA, no (%)	1 (2.9)	19 (22.4)	20 (16.7)
Column total, no (%)	35 (100)	85 (100)	120 (100)
Detection of pepA Among Paired TBB Reports During the Study Period*			
Covariate	**TBBs with ACR ≥A1 (n = 49)**	**TBBs with A0 Grade (n = 279)**	**Row Total, no (%)**
Detectable pepA, no (%)	36 (73.5)	164 (58.8)	200 (61)
Undetectable pepA, no (%)	13 (26.5)	115 (41.2)	128 (39)
Column total, no (%)	49 (100)	279 (100)	328 (100)

*Pathology reports reporting inadequate sample (i.e., AX) were excluded. Abbreviations: ACR, acute cellular rejection; BAL, bronchoalveolar lavage; LTR, lung transplant recipient; LTx, lung transplantation; pepA, activated pepsin; TBB transbronchial biopsy.

Further, an ROC analysis was performed to explore the role of pepA as a quantitative biomarker to detect ACR using data from all BAL fluid samples and paired TBBs (Figure 1). The area under the curve (AUC) for pepA concentration in BAL fluid was 0.587 (CI95: 0.503-0.671, P = 0.043), and the optimal cut-off to detect ACR was >2.45 ng/mL, determined by a Youden index of 0.206. At this threshold, the sensitivity was 63.3% and the specificity was 57.3%.

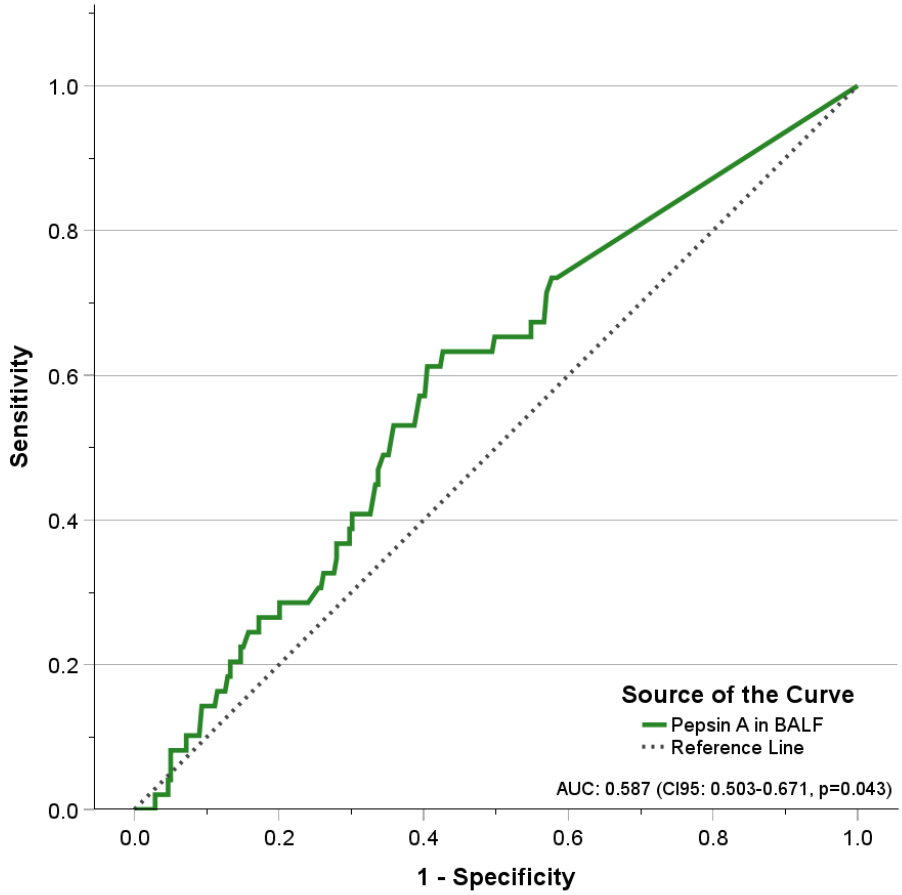

Figure 1 Receiver operating curve for pepsin A concentrations in bronchoalveolar lavage fluid samples for prediction of acute cellular rejection. Abbreviations: AUC: area under the curve; BALF: bronchoalveolar lavage fluid; CI95: 95% confidence interval.

3.3 Microbiological Assessment

Of the 349 BAL samples, 116 (33.2%) had a positive de novo microbial culture or viral PCR test result: 55 (47.4%) had positive fungal cultures, 45 (38.8%) had positive bacterial cultures, and 16 (13.8%) had a positive viral PCR test. In 6 (5.2%) BAL samples, multiple microorganisms were identified. Of the 35 different isolated microorganisms, *Pseudomonas aeruginosa* (n = 15 [12.9%]) and *Aspergillus spp.* (n = 17 [14.6%]) were the most common, and 10 of the 35 microorganisms identified are typically found in the GI tract. Of the 116 positive BAL samples, 27 (23.3%) contained microorganisms that are typically found in the GI tract.

The median pepA concentration was higher in samples with concurrent isolation of microorganisms than in those without (3.0 vs. 0.9 ng/mL, P = 0.020). Although BAL fluid samples with isolation of GI-related microorganisms had a higher pepA concentration than those with isolation of non-GI microorganisms, the difference did not reach statistical significance (4.4 vs. 2.2 ng/mL, P = 0.267). Table 4 summarizes the microbiological assessments and findings, and Supplementary material Table S3 summarizes the frequency of isolated microorganisms.

Table 4 Bronchoalveolar lavage characteristics and histological acute cellular rejection grading according to microbiological assessment.

Covariate	BAL with Negative Isolations (n = 233)	BAL with Positive Isolations (n = 116)	*P-value*	BAL with Isolation of Non-GI-related Micro-organisms, (n = 89)	BAL with Isolation of GI-related Micro-organisms, (n = 27)	*P-value*
Time between LTx and BAL, days	188 (86-296)	183 (93-352)	0.485	168.5 (93-320)	270 (65-382)	0.208
Pepsin A, ng/mL	0.9 (0-6.7)	2.95 (0-8.15)	**0.020**	2.2 (0-7.3)	4.4 (1.4-8.5)	0.267
Paired TBB with ACR ≥A1, No. (%)	30 (12.9)	19 (16.4)	0.459	17 (19.1)	2 (7.4)	0.151

Data presented as median (interquartile range) unless otherwise specified. Assessment of differences between ACR grades was conducted using the Mann-Whitney U test or the Chi-Square test as appropriate. Bold indicates statistically significant p-values ($p < 0.05$). Abbreviations: ACR, acute cellular rejection; BAL, bronchoalveolar lavage; GI, gastrointestinal; LTx, lung transplantation; TBB, transbronchial biopsy.

3.4 Confounding Factors

3.4.1 Time between LTx and BAL

The time between LTx and BAL showed no correlation with the BAL pepA concentrations ($r = 0.0544$, $P = 0.312$).

3.4.2 SARS-CoV-2 Detection

For this study, the analysis of SARS-CoV-2 isolation from the BAL samples was omitted given its current worldwide high incidence and prevalence.

4. Discussion

CLAD, which can be precipitated by episodes of ACR and chronic aspiration, is the most common cause of death among LTRs [6-8]. Our study found that detectable pepA in BAL fluid is common among LTRs (83.3% during the study period), and patients presenting with detectable pepA at any point during the study period were more likely to have had at least one episode of ACR (OR 9.79 [CI95 1.26-79.26]). Moreover, we identified a trend toward higher pepA and total protein concentration as the histological ACR grade increased and established an optimal pepA concentration cut-off of >2.45 ng/mL to detect any grade of ACR with a sensitivity of 63.3% and specificity of 57.3%. Lastly, the median pepA concentration was higher among BAL samples with concurrent microorganism isolation than those without (3.0 vs. 0.9 ng/mL, $P = 0.020$); however, there was no difference in the median pepA concentration between samples with isolation of

organisms typically found within the GI tract and those with organisms typically found outside of the GI tract (2.2 vs. 4.4 ng/mL, P = 0.267).

In 2005, Ward et al. [19] documented for the first time a significantly higher median pepA concentration in BAL fluid samples from a cohort of 13 LTRs than in samples from a control group of 4 healthy subjects (109 ng/mL vs. <1 ng/mL, P = 0.003). The authors confirmed their findings in a study comparing BAL pepA levels among 36 LTRs, 4 healthy controls, and 17 subjects with unexplained chronic cough [20]. Importantly, 2 of the controls had undetectable levels of pepA, whereas pepA was detected in very low concentrations (<2.3 ng/mL) in the other two. Additionally, they showed that pepA concentrations were higher in stable LTRs, those with relevant ACR (defined as grade ≥A2), and those who developed bronchiolitis obliterans syndrome (BOS) than in control subjects. The authors also documented that the concentration of pepA tended to be higher in LTRs with more severe grades of ACR, which supports our findings; however, in our study, using a less restricted cut-off for relevant ACR (≥A1), we demonstrated that LTRs with a detectable level of pepA were more likely to have an ACR episode during the study period. Further, LTRs without detectable pepA in surveillance bronchoscopies were highly unlikely to present with ACR, providing a strong negative predictive value. Interestingly, Stovold and collaborators [20] also reported that pepA levels were significantly higher among LTRs with ACR >A2 than in healthy controls or non-LTx patients with GERD and chronic cough, suggesting that pepA may be a reliable biomarker of aspiration under specific circumstances.

Another study in 2011 by Fisichella et al. [21] found a significant relationship between higher levels of pepA and the severity of ACR among 64 LTRs (P = 0.023). They also found that LTRs with any detectable levels of pepA had a faster progression to BOS than those with undetectable levels of pepA. Moreover, the authors reported that pepA concentrations were higher in BAL samples from LTRs regardless of esophageal acid exposure, and also that pepA concentrations were significantly lower among LTRs who underwent antireflux surgery. Two years later, the authors increased the sample and analyzed 257 BAL fluid samples prospectively collected from 105 LTRs; the results showed that pepA levels were higher in patients with BOS [22]. Contrary to these findings, Blondeu et al. [23] found that pepA concentrations did not correlate with BOS among a cohort of 45 LTRs. However, they did report a high prevalence of detectable pepA concentrations in BAL fluid samples of LTRs (100%), which is similar to the findings in our study (83.3%). These findings show that pepA may function primarily as an indicator of aspiration rather than being the causative agent of allograft damage.

In our study, microorganisms were isolated from 33.2% of the BAL fluid samples; *Aspergillus spp*. was the most common microorganism (17 of 116 isolations, 14.6%), and *P. aeruginosa* was the most common bacteria (15 of 116 isolations, 12.9%), which is in line with the study by Stovold et al. [20] that reported microorganism isolation from 19.4% of BAL samples, mostly *P. aeruginosa*. Moreover, 85.7% of BAL fluid samples from which microorganisms were isolated also had detectable levels of pepA, which is similar to our findings. Recently, in 2022, Schneeberger et al. [24] analyzed 268 BAL samples from 75 LTRs and classified the bacterial composition into three main microbial community types; the authors reported that LTRs with GERD had significantly more bacterial variability within the first year after transplant than those without GERD, and LTRs who underwent antireflux surgery had a decrease in bacterial density. Although a high microbiological variability was found in our study, and pepA concentrations were higher in BAL samples with positive isolation results, we did not identify correlations between pepA concentrations and the microbiological isolation of GI-

related microorganisms, suggesting that aspiration of gastric contents may not always be associated with the detection of pepA or that not all infections caused by GI-related microorganisms are necessarily associated with aspiration of gastric contents in LTRs.

Early detection of silent aspiration of duodenogastric contents can enable prompt treatment to prevent subsequent aspiration-induced allograft injury leading to complications like ACR or BOS; hence, it has been hypothesized that detection of pepA in BAL fluid may be a valuable biomarker in this scenario. Nevertheless, the routine use of this endopeptidase has several limitations and drawbacks. First, there is a lack of a standardized bronchoscopy technique to acquire BAL fluid samples with better sensitivity for pepA detection. In our case, and most of the published studies, the analyzed samples were taken from the right middle lobar bronchus [21-23]; however, anatomically, the aspirated contents are more likely to be located in the right lower lobar bronchus. Moreover, interpreting pepA concentrations in BAL fluid is challenging due to the recent detection of pepA in organs not related to the respiratory or digestive system (e.g., kidney or parotid gland) as well as potential cross-reactivity or cross-production reactions associated with pepsinogen isoforms (i.e., type C pepsinogen) that are generally produced by type 2 pneumocytes for surfactant degradation [15, 25].

Perhaps the value of pepA measurement in BAL samples lies in an adjustment of the threshold to treat patients with suspected ACR (e.g., symptomatic LTRs with an AX pathology report); however, there is no consensus regarding the cut-off for pepA concentration in BAL fluid. In most cases, the manufacturer or the laboratory determines the cut-off (i.e., depending on the technique, sample type, and diagnostic target); however, an optimal cut-off point based on clinical relevance to avoid overdiagnosis remains unclear [15, 25]. Notably, the ROC analysis in this study indicates that a pepA concentration >2.45 ng/mL had a sensitivity of 63.3% and a specificity of 57.3% to detect ACR ≥A1 grade, which could provide clinical guidance when ACR is suspected without pathological confirmation. Irrespective of pepA concentration, all patients with any grade of ACR detected by TBB receive treatment at our center. In the case of grade A1, management includes an oral prednisone burst for 5 days followed by the baseline home dosing. In cases of ACR ≥2, intravenous pulses of methylprednisolone (5-10 mg/kg × 3 days) are the primary treatment of choice. In both cases, the immunosuppressive regimen is adjusted or augmented as necessary based on drug monitoring, and a follow-up TBB is performed 1 month after treatment completion.

On the other hand, the real cause of allograft damage is aspiration and not necessarily aspiration of gastroesophageal reflux contents. For this reason, alternative BAL fluid biomarkers to document aspiration and predict ACR or CLAD have been proposed (e.g., IL-6, IL-8, IL-12p70 IL-15, IL- 17, IgG2/IgG1 ratio, basic fibroblast growth factor, tumor necrosis factor-alpha and alpha-1-antitrypsin, soluble RAGE, among others) [22, 26, 27]. However, an evidence-based consensus is yet to be established. To date, pepA measurement in BAL fluid continues to be invasive (i.e., bronchoscopy is always necessary regardless of the patient's symptoms); therefore, the characterization of a non-invasive biomarker (e.g., in serum or sputum) is highly desirable. The detection of antibodies against lung self-antigens collagen-V or k-alpha-1-tubulin in serum, which has been correlated with lower CLAD-free survival among LTRs with GERD, may hold the most promise [8, 28, 29].

4.1 Study Limitations

Our study has several limitations besides the retrospective, single-center design. First, approximately 35.8% of patients who underwent LTx during the study period were excluded due to failure to measure pepA in BAL fluid at any subsequent TBBs. The reasons for the lack of pepA assessment include unscheduled bronchoscopies for graft dysfunction and studies performed by different transplant pulmonologists, resulting in a potential selection bias. However, by including only bronchoscopies performed by a single pulmonologist, we reduced the heterogeneity of the BAL fluid sampling technique. Second, there is no consensus on the best pepA measurement technique or optimal normal cut-off values, which significantly limits the external validity of our findings and the use of the established optimal pepA concentration cut-off of >2.45 ng/mL to detect ACR. Third, the clinical condition of the recipients at the time of bronchoscopy was not documented, thus, the detection of higher concentrations of pepA and total proteins (despite its statistical significance) may be associated with microbial density or other conditions related to tissue inflammation. Fourth, our data collection did not include the history of antireflux surgery, thus, we could not compare pepA concentrations in this specific subgroup of recipients. In addition, we did not include an analysis of correlations between reflux parameters (i.e., DeMeester score or acid exposure time) and the concentrations of pepA or histological grades of ACR because the bronchoscopies were performed at different times than the pH monitoring studies, which significantly limits the interpretation of results. Finally, factors such as the underlying diagnosis leading to LTx, use of certain medications, and other patient comorbidities were not assessed, thus limiting the understanding of the use of pepA as a biomarker in very specific scenarios. Nevertheless, to the best of our knowledge, this is the largest analysis of pepA BAL fluid concentrations and their association with ACR among LTRs.

5. Conclusions

In summary, our study confirmed a high prevalence of detectable pepA in BAL fluid among LTRs. Almost all patients presenting with ACR had detectable pepA at some point after LTx, and those with the most severe ACR grades presented with higher concentrations of pepA in the concomitant BAL. We also established an optimal pepA BAL fluid concentration cut-off of >2.45 ng/mL with a sensitivity of 63.3% and specificity of 57.3% for the detection of ACR, which could lend evidence to the treatment of patients with suspected ACR but an inconclusive pathology report. Finally, we identified higher pepA concentrations in samples from which microorganisms were isolated. Although detection of pepA in BAL fluid after LTx is common among LTRs presenting with ACR at any point after LTx, the use of this endopeptidase as a surrogate biomarker of lung damage associated with gastric aspiration in LTRs has several pitfalls, and caution is recommended when interpreting these results. Key issues to resolve include the best technique for BAL sampling and processing, standardization of pepA concentration cut-off values for different diagnoses (e.g., ACR, CLAD, silent aspiration), and its clinical relevance. It is clear that an association exists between aspiration and allograft injury, which represents an exciting opportunity to improve LTR outcomes; however, further studies are strongly encouraged to evaluate the role of other potential and more specific non-invasive biomarkers for detecting silent aspiration and related LTx complications.

Abbreviations

ACR,	acute cellular rejection
AMR,	antibody-mediated rejection
AUC,	area under the curve
BAL,	bronchoalveolar lavage
BOS,	bronchiolitis obliterans syndrome
CLAD,	chronic lung allograft dysfunction
GERD,	gastroesophageal reflux disease
GI,	gastrointestinal
LTR,	lung transplant recipient
LTx,	lung transplantation
OR,	odds ratio
pepA,	activated pepsin
ROC,	receiver operating characteristic
TBB,	transbronchial biopsy

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Author Contributions

Conception and design: A.L., S.M And A.A. Data collection: A.L and A.A. Assembly of data, data analysis, and drafting of the manuscript: A.L, A.A, D.S and S.M. Critical revision of the manuscript: A.L, D.S, S.M and A.A. All the authors approved the final manuscript.

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Competing Interests

None of the authors have a commercial or financial conflict of interest to disclose.

Data Availability Statement

The data analyzed in this study is stored in a secure Research Electronic Data Capture system (RedCAP) that can be queried for future studies; however, it cannot be shared outside of those authorized as research staff per protocol. Access to this dataset requires IRB approval; if needed, direct to the corresponding author.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.
2. Table S2: Inclusion and exclusion criteria.
3. Table S3: Classification and absolute frequency of microorganisms isolated from bronchoalveolar lavage samples (n = 116) from lung transplant recipients.

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