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ORIGINAL CLINICAL SCIENCE

Assessing the role of phosphorylated S6 ribosomal protein in the pathological diagnosis of pulmonary antibody-mediated rejection

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KEYWORDS:
antibody-mediated rejection;

BACKGROUND: Pulmonary antibody-mediated rejection is still a challenging diagnosis as C4d immunostaining has poor sensitivity. Previous studies have indicated that the phosphorylated S6 ribosomal protein, a component of the mammalian target of rapamycin (mTOR) pathway, is correlated

Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection; BAL, bronchoalveolar lavage; DAD, diffuse alveolar damage; DSA, donor-specific antibodies; IHC, immunohistochemistry; INF, infection; IRI, ischemia-reperfusion injury; ISHLT, International Society of Heart and Lung Transplantation; LASHA, lung allograft standardized histological analysis; OP, organizing pneumonia; p-S6RP, phosphorylated s6 ribosomal protein; TBB, transbronchial biopsies; UCLA, University of CA Los Angeles

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lung transplantation;
phosphorylated s6 ribosomal protein (p-S6RP);
mammalian target of rapamycin (mTOR) pathway;
antibody-mediated rejection (AMR)

with de novo donor-specific antibodies in lung transplantation. The objective of this study was to evaluate the phosphorylation of S6 ribosomal protein as a surrogate for antibody-mediated rejection diagnosis in lung transplant patients.

METHODS: This multicentre retrospective study analyzed transbronchial biopsies from 216 lung transplanted patients, 114 with antibody-mediated rejection and 102 without (19 with acute cellular rejection, 17 with ischemia/reperfusion injury, 18 with infection, and 48 without post-transplant complications). Immunohistochemistry was used to quantify phosphorylated S6 ribosomal protein expression in macrophages, endothelium, epithelium, and inter-pathologist agreement was assessed.

RESULTS: Median phosphorylated S6 ribosomal protein expression values were higher in antibody-mediated rejection cases than in controls for all cell components, with the highest sensitivity in macrophages (0.9) and the highest specificity in endothelial expression (0.8). The difference was mainly significant in macrophages compared to other post-lung transplantation complications. Inter-pathologist agreement was moderate for macrophages and endothelium, with higher agreement when phosphorylated S6 ribosomal protein expression was dichotomized into positive/negative. The inclusion of phosphorylated S6 ribosomal protein in the diagnostic algorithm could have increased antibody-mediated rejection certainty levels by 25%.

CONCLUSIONS: The study supports the role of the mTOR pathway in antibody-mediated rejection-related graft injury and suggests that tissue phosphorylation of S6 ribosomal protein could be a useful surrogate for a more accurate pathological diagnosis of lung antibody-mediated rejection.

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Antibody-mediated rejection (AMR) in the pulmonary allograft is a relatively new and evolving concept that is likely responsible for important graft failure after lung transplantation in a subset of patients. However, diagnosis of AMR in lung biopsies is still challenging.

Several consensus statements have highlighted the importance of a multidisciplinary approach for the final diagnosis of AMR,[1,2] with pathologists playing an important role in the multi-specialist team. Different morphological features have been described as suggestive of AMR with a more frequent occurrence of diffuse alveolar damage (DAD), organizing pneumonia (OP), different degrees of capillary inflammation, endotheliitis,[3,4] and alveolar septal widening,[5] all changes that are unfortunately not specific. To date, the diagnosis of lung AMR shows three levels of certainty and is based on donor-specific antibody (DSA) assessment, lung histology, C4d immunostaining, and is defined as clinical or subclinical if signs/symptoms of respiratory dysfunction are present.[2] In particular, C4d positive staining is considered when at least 50% of capillaries are marked. Unfortunately, there are important weaknesses in this methodology due to technical and interpretation problems (e.g., infrequent diffuse staining, autofluorescence of elastic fibers, poor inter-pathologist agreement)[3] leading to an underdiagnosis of AMR and/or levels of AMR certainty. Furthermore, there is growing acceptance that graft injury may occur via complement-independent antibody-mediated mechanisms (i.e., C4d-negative AMR).[6] Thus, there is a strong need to refine the morphological evaluation with potentially more sensitive AMR surrogates.

Several in vitro studies have shown that after ligation of both classes I and II HLA antibodies on the surface of different cell components, mainly endothelial, there is a prosurvival signaling cascade via the mTOR pathway.[7–10] S6 ribosomal protein, which becomes phosphorylated following the ligation of HLA molecules, is one of the activated signaling molecules. The antibody specific to this protein (p-S6RP) has been shown to be an effective biomarker of pathological AMR and DSA status in cardiac transplant recipients.[11–14] However, the utility of p-S6RP immunostaining in the setting of pulmonary AMR has not been deeply investigated. High expression of this protein has been reported in one anecdotic case in which histological findings were highly suggestive of AMR, despite a negative C4d staining.[15] A very recent pilot study of Fishbein's group showed a positive relationship between pneumocyte and alveolar macrophage expression of p-S6RP and the presence of circulating DSA[16] but, to date, there are no conclusive studies about the sensitivity and specificity of this biomarker in the multidisciplinary diagnosis of lung AMR.

While the role of endothelial cells in lung AMR has already been demonstrated, the importance of other components such as epithelial and inflammatory is currently under debate. Moreover, to the best of our knowledge the role of mTOR pathway in lung AMR different cell types has never been studied.

The aim of the study was to establish the sensitivity and specificity of p-S6RP immunostaining expression in post-transplant biopsies from patients with AMR in comparison with cases affected by other post-transplant complications, distinguishing pneumocytes, macrophages and endothelial cells. Each case of AMR was categorized (AMR definite, probable, and possible) after a multidisciplinary discussion with experienced transplant pathologists, pulmonologists, and immunologists according to the current ISHLT consensus statement.

Materials and methods

Study design and population

This multicenter retrospective cohort study included 216 transbronchial biopsies (TBBs), which were obtained from patients who consecutively underwent lung transplantation in the 2009–2018 period in different centers [University of California, Los Angeles (UCLA) (Los Angeles, California, USA), Padova University Hospital (Padova, Italy), Hôpital Marie Lannelongue (Le Plessis Robinson, France), University of Iowa (Iowa City, Iowa, USA), Rigshospitalet (Copenhagen, Denmark) and University Hospital of Strasbourg (Strasbourg, France)]. In particular, for AMR cases each Center reviewed post-transplant TBBs obtained from all patients with clinical/pathological diagnosis or suspicion of AMR and only one biopsy per patient was included in the study, following the inclusion criteria: (1) informed consent; (2) patients with complete clinical, immunological, and pathological records (http://lungtransplant.dctv.unipd.it/amr/index.php; Figure S1, SDC, https://links.lww.com/TP/B709); (3) biopsy obtained at the first AMR diagnosis and necessarily within the first three years after lung transplantation; (4) tissue adequacy (at least 5 pieces of alveolated parenchyma); (5) absence of preformed DSA; (6) no previous diagnosis/treatment for AMR (Figure 1). Furthermore, each center was asked to provide an equal number of controls in the same enrollment period with the following inclusion criteria: (1) informed consent; (2) patients with complete clinical, immunological, and pathological records, well-matched with cases in terms of age, sex and native diseases; (3) biopsy obtained within the first three years after lung transplantation without any post-transplant complications (negative), with acute cellular rejection (ACR), with ischemia/reperfusion injury (IRI) or with infection (INF); (4) biopsy negative for any post-transplant complications from the previous control, to avoid any influence related to therapeutic changes; (5) tissue adequacy (at least 5 pieces of alveolated parenchyma); (6) absence of preformed/de novo DSA. In the control group, we excluded cases with multiple complications such as ACR + INF, IRI + ACR, IRI + INF. (Figure 1).

The study was designed in accordance with the Helsinki declaration and is compliant with the ISHLT Ethics statement. All patients gave informed consent for research purposes. The Institutional Ethics committee approved the study (AOP2860). Immunosuppression and prophylaxis treatments were given following the protocols of each center. Patients were followed with a scheduled protocol of surveillance consisting of TBB and bronchoalveolar lavage (BAL).[17] At each time point, clinical assessment consisted of spirometry in conjunction with blood gas analysis, measurement of immunosuppressive drug levels, chest radiographs, and/or computed tomography. DSA screening was done at the time of the TBB or ± 2 weeks. Chronic lung allograft dysfunction (CLAD) development and survival were updated in May 2023. The patient characteristics are presented in Table 1.
Sample selection: We intentionally designed our study to minimize the impact of heterogeneity across the six centers. Each participating center was tasked with blind selection of its AMR samples and a similar number of control samples. This approach ensured that AMR and control samples from each center were contemporaneous, thereby reducing temporal biases or shifts in patient demographics or treatment protocols that might have varied over time or over centers.

Histological evaluation: To further mitigate the influence of site-specific variation, all histological evaluations were centralized. They were conducted in two centers (Padova University Hospital and UCLA University) that had already standardized and shared protocols of analysis. These protocols were validated before the study with the blind analysis of shared (the same) samples and agreement evaluation by three experts. This centralized approach ensured consistent handling, processing, and assessment of all biopsy samples, eliminating inter-laboratory variability that might arise from using different equipment, reagents, or technicians.

DSA assessment

Peripheral blood DSA analysis was performed on all index patients at or close to the time of the reference biopsy. Anti-HLA Class I and Class II IgG antibodies were evaluated with protocols specific for each center.

Multidisciplinary discussion

All cases were discussed in a multidisciplinary team (pathologist, immunologist, pulmonologist) to establish the precise AMR levels of certainty. A subgroup of the study population came from a previous multicenter study (48 cases). Subclinical AMR was excluded.

Immunohistochemistry

Immunohistochemistry (IHC) for both p-S6RP and pS6K was performed in all the TBBs following the antibody manufacturer's protocol. Immunostaining was centralized in 2 centers that have had previous experience with the use of this marker (Padova and UCLA Translational Pathology Core laboratories).[15,16] For pS6K, different protocols were used but staining was negative in almost all cases, thus the study was focused only on p-S6RP. Briefly 3–4 μm-thick sections were processed for IHC analysis using antibody anti-p-S6RP (1:300, Ser235/236 Antibody #2211, Cell Signaling Technology) in the Leica Bond Autostainer (Leica Microsystems Srl, Wetzlar, Germany) according to the manufacturer's protocol. Finally, the sections were counterstained with Mayer's haematoxylin. Immunostaining was evaluated in different cell types (macrophages, endothelium, epithelium) using a scoring system from 0 to 3 (0: no staining; 1: focal staining, present in only one sample; 2: multifocal staining, present in half the samples; 3: diffuse staining, present in all samples). A total score obtained combining all 3 cell type expression values was calculated. Single cell expression values and total scores were dichotomized into positive (≥1) and negative (0), but also into positive (3) and negative (< 3). We considered a total score of 7 as a good cutoff point, as indicative of p-S6RP expression in all cell components, with at least one of them with a score 3 and one with at least a score 2 (3 + 3 + 1 or 3 + 2 + 2). Four expert lung pathologists evaluated the cases (FC, KW, FL, and GF). The inter-pathologist agreement between 2 pathologists was also evaluated. Background staining of respiratory epithelial cells was ubiquitous when airway epithelium (sloughed or intact) was present in the biopsies and it was considered our internal positive control. The evaluation was restricted only to strong cytoplasmic staining of the considered cell types. For endothelial expression, staining was evaluated in capillaries, venules, and small arteries. In a subset of cases to prove the p-S6RP expression in the specific cell types, multiplex immunofluorescence with Opal 3-Plex Detection Kits (Akoya Biosciences) with the following primary antibodies was performed according to the

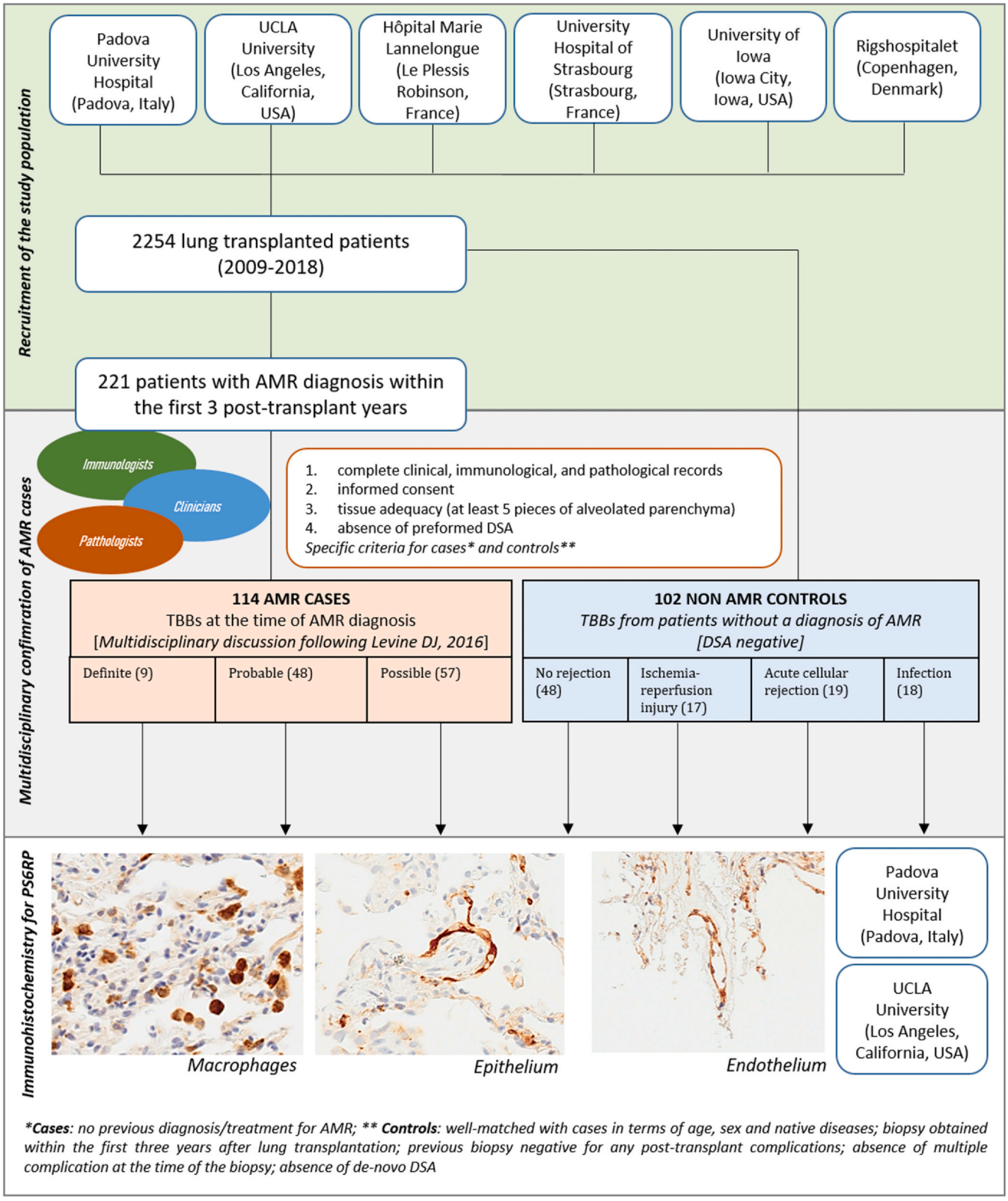


Figure 1 CONSORT diagram. *Negative: biopsies without any post-transplant complication. ACR, acute cellular rejection; AMR, antibody-mediated rejection; INF, infection; IRI, ischemia-reperfusion injury; LTx, lung transplantation; p-S6RP, phosphorylated S6 ribosomal protein; UCLA, University of CA Los Angeles.

manufacturer's protocol: anti-CD68/anti-AE1AE3/anti-p-S6RP (for co-localization of p-S6RP with macrophages and epithelial cells) and anti-CD31/anti- p-S6RP (for co-localization of p-S6RP with endothelial cells) (data not shown).

Statistical analysis

The data in the study is presented as the median (interquartile range) and compared using Fisher's exact test, Pearson's

Table 1 Main Clinical-Pathological Characteristics of the Study Population

Characteristic	AMR cases ($N = 114$)	non-AMR controls ($N = 102$)	p-value
Age (years)	50 (29,62)	56 (37,62)	
Sex (females)	51%	38%	
Native disease			
CF	17%	18%	
IPF	25%	25%	
Other	58%	57%	
Smoking history (smokers)	39%	21%	
Donor age (years)	36 (23,48)	42 (24,54)	
Donor sex (females)	40%	40%	
Type of transplantation (bilateral)	75%	81%	
Mean ischemic time (min)	269 (235,332)	288 (226,366)	
Timing of the biopsy (months)	81 (10,356)	85 (0225)	
DSA (positive)	98%	0%[a]	
DSA type – Class I	9%	-	
DSA type – Class II	60%	-	
DSA type – Class I and II	31%	-	
STATUS (deceased) – RMST (95% CI)			
2 years	1.78 (1.69–1.87)	1.71 (1.61–1.82)	0.378
5 years	3.61 (3.29–3.92)	3.60 (3.25–3.96)	0.991
10 years	5.60 (4.89–6.31)	6.04 (5.21–6.86)	0.431
CLAD – RMST (95% CI)			
2 years	1.76 (1.67–1.85)	1.96 (1.91–2.00)	< 0.001
5 years	3.46 (3.12–3.80)	4.70 (4.50–4.91)	< 0.001
10 years	5.44 (4.66–6.22)	8.42 (7.67–9.17)	< 0.001

AMR, antibody-mediated rejection; CF, cystic fibrosis; CLAD, chronic lung allograft dysfunction; DSA, donor specific antibodies; IPF, Idiopathic Pulmonary Fibrosis; RMST, restricted mean survival time.

Continuous variables are expressed as median (Q1, Q3), while categorical are expressed as %.

[a]DSA positivity was an exclusion criteria for the control group, RMST: restricted mean survival time (years).

Chi-square test or Wilcoxon rank sum test, depending on the type of variable. The dataset was complete, so no missing data imputation was performed. The sensitivity, specificity, and other performance parameters were calculated and reported using the report ROC R package.[18] The agreements were assessed using Cohen's Kappa. ROC plots were generated using the pROC R package.[19] A total score of ≥7 was chosen as suggestive of AMR diagnosis and was considered, in addition to C4d value, to evaluate the change in AMR levels of certainty. This cut-off was also used for overall survival and CLAD-free survival analyses. The improvement in AMR levels of certainty was assessed by comparing "probable" to "definite" and "possible" to "probable." Significance was set at $p < 0.05$, and R 4.2.2 was used for analysis and plotting.[20] To investigate the relationship between the p-S6RP score and the overall survival or CLAD-free survival, we conducted a survival analysis using Kaplan-Meyer (KM) curves (log-rank test) and the Cox proportional hazards regression model. A total score of ≥7 was chosen as suggestive of AMR diagnosis and used as cut-off value for prognostic stratification. Covariates included in the Cox regression were age, sex, biopsy timing (i.e., the time intercurred from the transplant to the biopsy), native disease, donor age, donor sex, donor smoking status, type of transplant (mono or bilateral), and ischemia time. Since donor data were not always available due to privacy laws, we had about 9% of missing data on our dataset. We imputed the missing data using a robust random-forest based method from the {randomForestSRC} R package v.3.2.2 (by Ishwaran H. and Kogalur U.B). Proportional hazards assumption of the Cox regression was checked inspecting the Schoenfeld residuals of each covariate, plotted against time.

Results

Study population

One hundred and fourteen patients had a diagnosis of clinical AMR (*AMR cases*) after a multidisciplinary team discussion following the most recent consensus report[2] and were classified as definite (9 cases), probable (48 cases), and possible (57 cases). One hundred and two patients did not have a diagnosis of AMR during their follow-up (*controls*), and the following TBB (1 for each patient) were included: 48 without any post-transplant complications (negative), 19 with acute cellular rejection (ACR), 17 with ischemia/reperfusion injury (IRI) and 18 with infection (INF). All the TBB of controls were chosen within the follow-up time considered in the inclusion criteria (within the first 3 years post-transplantation). In 30/114 (26%) AMR cases, ACR was concomitantly detected (mixed rejection). AMR cases and controls were well-matched in terms of demographic and clinical characteristics (Table 1).

Immunohistochemistry findings

P-S6RP immunostaining was mainly cytoplasmic and detected in all the three considered cell types, particularly in macrophages, with a median (Q1–Q3) total score of 3 (1–6)

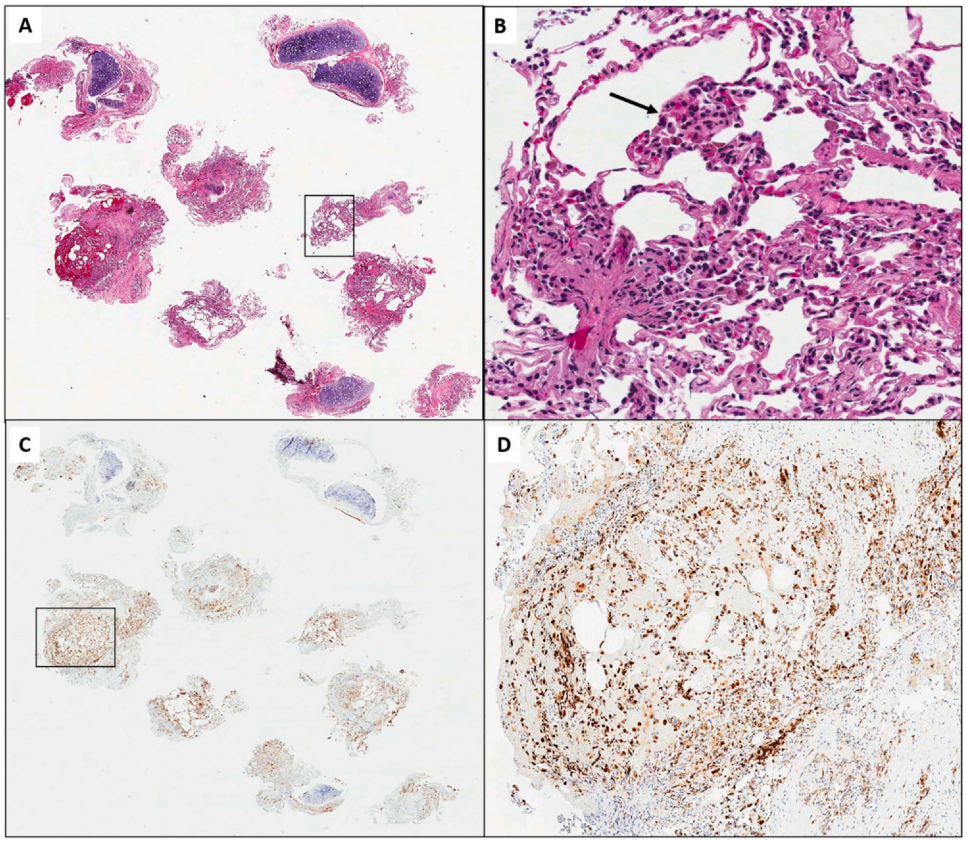


Figure 2 Emblematic AMR case, classified as probable for C4d negativity: haematoxylin and eosin panoramic view (A) and close-up of capillaritis (B); p-S6RP immunohistochemistry: panoramic view (C) and close-up showing strong immunostaining, particularly in macrophages and epithelial cells (D). AMR, antibody-mediated rejection; p-S6RP, phosphorylated S6 ribosomal protein.

(Figure 2). No significant differences were found among the three AMR levels of certainty (median, Q1–Q3: 4, 1–6 in definite, 2.5, 1–6 in probable and 6, 2–7 in possible). Focusing on immunological data, no differences were found in the different DSA type. Interestingly, C4d staining was positive in 24/114 AMR cases (21%) and negative in 90/114 (79%). The 2 groups of patient were not different in the p-S6RP expression, for every cell types and considering the total expression values. In cases with C4d negativity, AMR levels of certainty were improved when considering p-S6RP total score ≥7 as suggestive of AMR (Figure 3).

AMR versus controls

When comparing the p-S6RP expression of AMR with all control samples, a total score obtained adding each cell type score resulted able to identify convincingly AMR samples ($p = 0.001$). In particular, higher scores were detected in macrophages ($p = 0.002$), endothelial cells ($p = 0.033$), and epithelium ($p = 0.014$). After dichotomization of p-S6RP values into positive (score ≥1) and negative values, differences were confirmed as significant (Table 2). In particular, macrophagic p-S6RP expression showed the highest sensitivity (0.9), whereas endothelial p-S6RP expression showed the highest specificity (0.8). Epithelial expression had a sensitivity of 0.6 and a specificity of 0.5 (Figure 4). When considering only p-S6RP score 3 as positive, we found higher specificity for all the cell components (0.7 for macrophages, 0.8 for epithelium, 1.0 for endothelium).

AMR versus negative samples

When the expression of the p-S6RP of AMR was compared with negative samples (samples without any post-transplant complications), the total score was satisfactory in identifying AMR samples ($p = 0.003$). In particular, higher scores were detected in macrophages ($p = 0.011$), endothelial cells ($p = 0.007$), and epithelium ($p = 0.005$). After dichotomization of p-S6RP values into positive (score ≥1) and negative, the differences were confirmed (Table S1). In particular, macrophagic p-S6RP expression showed the highest sensitivity (0.8), whereas endothelial p-S6RP expression showed the highest specificity (0.9). Epithelial expression had a sensitivity of 0.6 and a specificity of 0.5 (Figure 5). When considering only p-S6RP score 3 as positive, we found higher specificity for all the cell components (0.7 for macrophages, 0.9 for epithelium, 1.0 for endothelium).

AMR versus other post-transplant complications

When comparing the p-S6RP expression of AMR with ACR samples, the total score obtained by adding each cell type score was satisfactory in identifying AMR samples

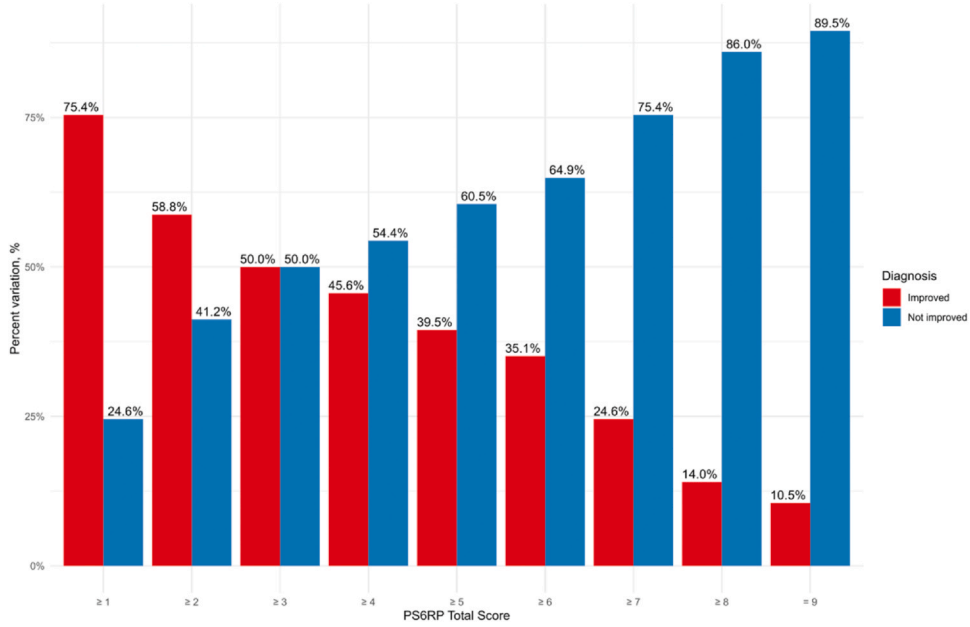


Figure 3 AMR levels of certainty improved after introducing p-S6RP total score in the evaluation. We calculated the percentage of improvement of AMR levels of certainty using p-S6RP total score in addition to C4d+. Improvement was assessed as the change in AMR category (i.e., "probable" to "definite" and "possible" to "probable"). The percentage represents the fraction of diagnosis that remained stable ("Not improved") or improved according to the p-S6RP total score. As expected, as the total score threshold increases, the number of improved diagnoses decreases. AMR, antibody-mediated rejection; p-S6RP, phosphorylated S6 ribosomal protein.

Table 2 P-S6RP Expression in AMR versus Control Samples

Variable	Controls ($N = 102$)	AMR ($N = 114$)	p-value
Macrophages (score)			0.002
0	26 (25%)	13 (11%)	
1	22 (22%)	28 (25%)	
2	23 (23%)	14 (12%)	
3	31 (30%)	59 (52%)	
Endothelium (score)			0.033
0	82 (80%)	73 (64%)	
1	12 (12%)	17 (15%)	
2	3 (2.9%)	9 (8%)	
3	5 (4.9%)	15 (13%)	
Epithelium (score)			0.014
0	54 (53%)	43 (38%)	
1	16 (16%)	21 (18%)	
2	15 (15%)	11 (10%)	
3	17 (17%)	39 (34%)	
Macrophages (positive)	76 (75%)	101 (89%)	0.007
Endothelium (positive)	20 (20%)	41 (36%)	0.008
Epithelium (positive)	48 (47%)	70 (61%)	0.035
Total score	2.00 (1.00, 4.75)	4.00 (1.00, 7.00)	0.001
Total score (positive)	38 (37%)	64 (56%)	0.006

AMR, antibody-mediated rejection; p-S6RP, phosphorylated S6 ribosomal protein.

($p = 0.024$) and was related to higher scores in the macrophages ($p = 0.013$), whereas in endothelium the difference was noted only after dichotomization ($p = 0.03$). Interestingly, when comparing samples with mixed rejection (ACR + AMR) with those with pure AMR, higher scores of p-S6RP expression were not only found in macrophages but also a trend in epithelial cells. After dichotomization, significance was confirmed (Table S2).

When comparing the p-S6RP expression of AMR with IRI samples, no differences were noted in any cell type, even using a dichotomized evaluation.

When comparing the p-S6RP expression of AMR with INF samples, higher total scores were detected in AMR samples ($p = 0.033$), mainly in macrophages, and especially when a dichotomized evaluation was used ($p = 0.025$).

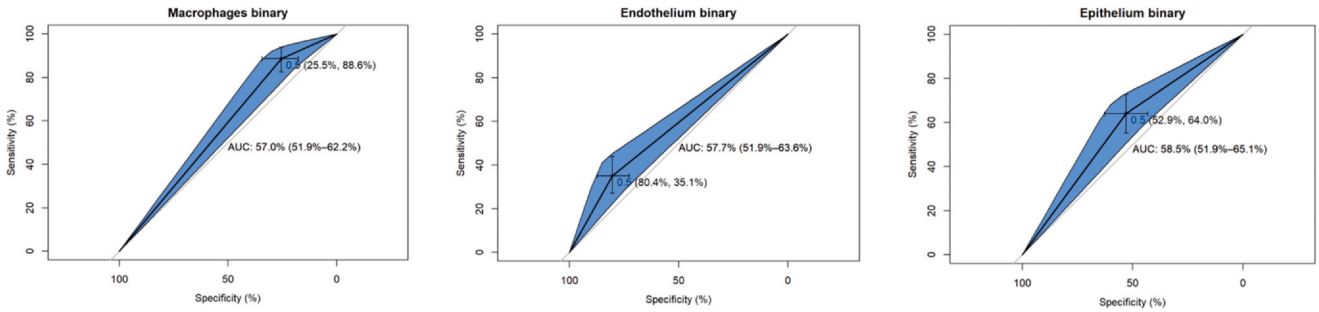
Box-plots showing the differences in p-S6RP expression between the different study groups are in Figure 6.

Inter-pathologist agreement

The evaluation of inter-pathologist agreement showed moderate values for macrophage and endothelium scores, while it was fair for epithelial ones. A higher agreement was found for dichotomic p-S6RP evaluations, even more so when considering only the p-S6RP score three as positive (Table 3).

Survival analyses

KM curves showed a significant difference in survival times: patients having a p-S6RP score of 7 or more had



	Sensitivity	Specificity	NPV	PPV	PLR	NLR	AUC	ACC	p
Macrophages	0.886	0.255	0.667	0.571	1.189	0.447	0.570	0.588	0.004
Endothelium	0.351	0.804	0.526	0.667	1.789	0.807	0.577	0.565	0.006
Epithelium	0.640	0.529	0.568	0.603	1.361	0.679	0.585	0.588	0.006

Figure 4 Sensitivity and specificity of macrophagic, epithelial and endothelial p-S6RP expression in detecting AMR cases versus controls. ACC, accuracy; AMR, antibody-mediated rejection; AUC, area under curve; NLR, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; PLR, positive likelihood ratio; p-S6RP, phosphorylated S6 ribosomal protein.

lower overall survival ($p = 0.021$) and CLAD-free survival ($p = 0.023$) times (Figure 7).

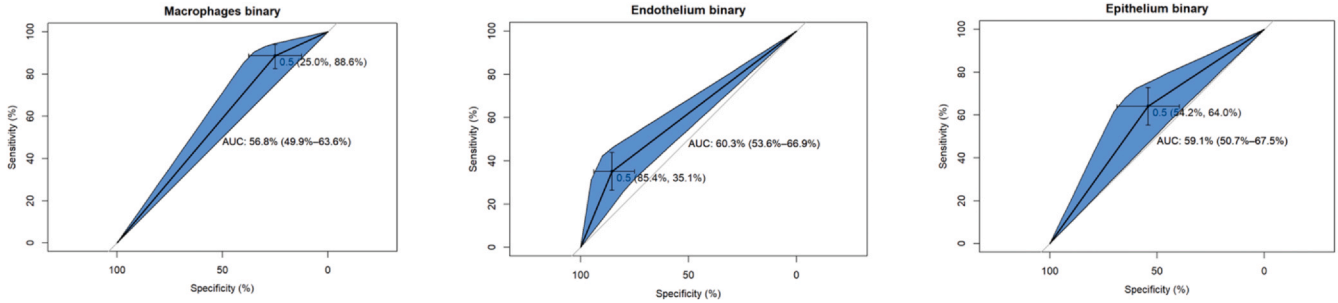
Cox regression for CLAD-free survival confirmed the KM results, having patients with a p-S6RP score of 7 or more a log (HR) = 0.48 (95% CI: 0.07–0.90, $p = 0.023$). Calculating the HR = exp (0.48) = 1.62 (95% CI: 1.01–2.45), this means that a p-S6RP score ≥7 increases the CLAD and survival risk by a mean of 62%, assuming the other variables in the model are held constant (Table S3).

Similarly, for the overall survival analysis, p-S6RP score ≥7 was associate with an increased risk of death of 80%, assuming the other variables in the model are held constant. Log (HR) = 0.59 (95% CI: 0.14–1.0, $p = 0.011$), exp (0.59) = 1.80 (95% CI: 1.12–2.84) (Table S4).

Discussion

Our data confirm an association between the diagnosis of AMR and the positive expression of p-S6RP in all cell types, especially in macrophages and pneumocytes. This association was evident when comparing AMR cases to control groups and was even more pronounced when compared to cases without any post-transplant complications. Similar findings were also observed when cases exhibited other post-transplant complications like ACR and INF, especially in specific cell types like macrophages.

The p-S6RP expression of our cases with mixed rejection (ACR + AMR) was higher in all cell components than in "pure" AMR cases. While the knowledge about



	Sensitivity	Specificity	NPV	PPV	PLR	NLR	AUC	ACC	p
Macrophages	0.886	0.250	0.480	0.737	1.181	0.456	0.568	0.698	0.986
Endothelium	0.351	0.854	0.357	0.851	2.406	0.760	0.603	0.500	0.004
Epithelium	0.640	0.542	0.388	0.768	1.397	0.664	0.591	0.611	0.016

Figure 5 Sensitivity and specificity of macrophagic, epithelial and endothelial p-S6RP expression in detecting AMR cases versus negative samples. ACC, accuracy; AMR, antibody-mediated rejection; AUC, area under curve; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value; p-S6RP, phosphorylated S6 ribosomal protein.

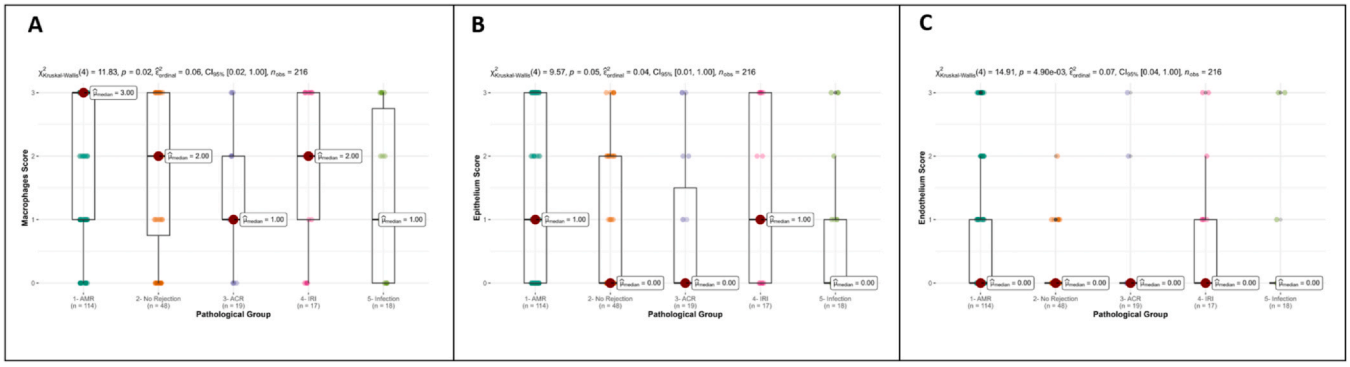


Figure 6 Box-plots showing the differences in p-S6RP expression among the different study groups (A: macrophages, B: epithelial cells, C: endothelial cells). p-S6RP, phosphorylated S6 ribosomal protein.

Table 3 Intra- and Inter-Pathologist Agreement in p-S6RP Expression Scoring

Characteristic	Inter-pathologist agreement
Macrophages score	0.522
Endothelium score	0.448
Epithelium score	0.282
Macrophages yes/no[a]	0.562
Endothelium yes/no[a]	0.574
Epithelium yes/no[a]	0.374
Macrophages yes/no[b]	0.77
Endothelium yes/no[b]	0.677
Epithelium yes/no[b]	0.617

p-S6RP, phosphorylated S6 ribosomal protein.
[a]p-S6RP values ≥ 1 (yes) versus p-S6RP value = 0 (no).
[b]p-S6RP value = 3 (yes) versus p-S6RP value < 3 (no).

mixed rejection (cellular + humoral) is progressively increasing in kidney transplantation[21] and recently also in heart transplants,[22] evidence in lung transplantation remains somewhat limited to anecdotal cases.[4,23] Mixed acute rejection is a clinicopathological entity that is difficult to accurately diagnose, and thus may be under-reported. This complication usually occurs early after transplant, is associated with worse outcomes, and reflects a complex interplay between cellular and humoral processes. Thus, it is not an unexpected finding to have detected the p-S6RP overexpression in all our cases with mixed rejection.

P-S6RP sensitivity was high in macrophages and epithelial cells and was low in endothelial cells that were more rarely positive but showed a high specificity (85%). A previous study reported similar data for the macrophagic and epithelial sensitivity in patients with positive de novo DSA.[16] The majority of our probable or possible AMR were HLA DSA positive. Thus, in the present case series, we were not able to prove whether p-S6RP was also expressed in the setting of AMR without HLA DSA. However, the Padova lung transplant team pioneered a strong p-S6RP immunostaining in post-transplant lung biopsies of a patient without HLA DSA but with high levels of anti-angiotensin II receptor type 1 and anti-endothelin-1 receptor type A antibodies.[15] Certainly, the value of this AMR

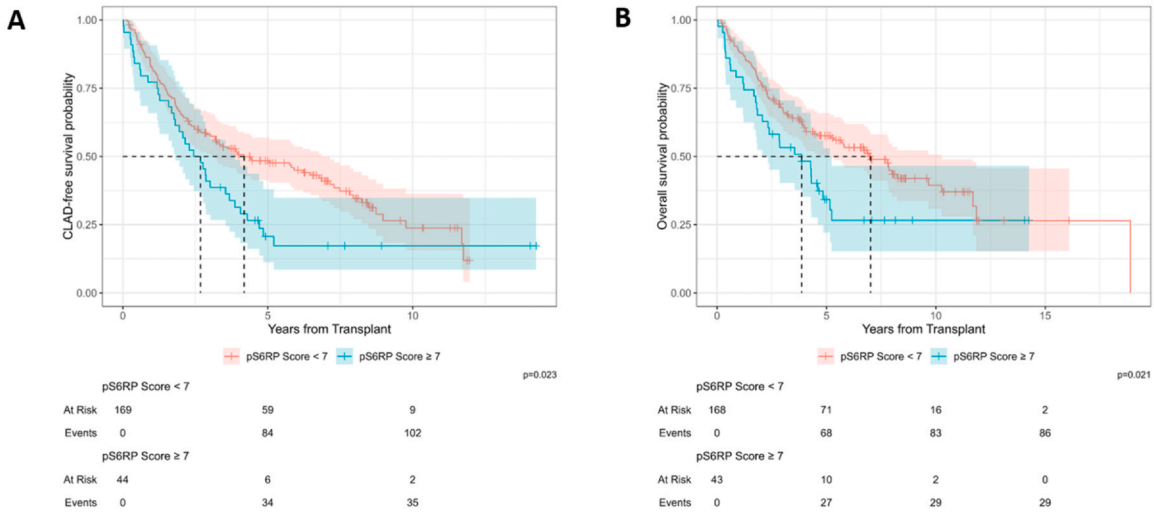


Figure 7 Kaplan Meyer curves about CLAD-free survival (A) and overall survival (B). A total score of ≥7 was chosen as suggestive of AMR diagnosis and used as cut-off value for prognostic stratification. AMR, antibody-mediated rejection; CLAD, chronic lung allograft dysfunction.

surrogate marker could have a higher diagnostic performance if this finding is confirmed on a larger case series of AMR with a considerable number of patients with non-HLA antibodies. It is unclear why p-S6RP expression in lung allografts with AMR is strongly expressed in several cell types, particularly alveolar macrophages, and epithelium, even if with low specificity in these cell types. mTOR plays a central role in a number of fundamental cell processes involving cell survival and proliferation. While we have extensive knowledge of mTOR pathway activity from several vitro studies, mainly coming from Reeds' group,[7–10] the exact function of most of the mTOR downstream signaling pathways in different grafts remains incompletely understood. The mTOR pathway is activated after various injuries promoting cell survival and proliferation[24]; thus, it is not surprising to detect strong expression of this marker in response to different graft insults. Alternative injuries such as ACR, IRI, and INF can easily be excluded taking into consideration a granular description as reported in LASHA grid [25] and several factors such as the timing of the TBB (e.g., for differential diagnosis of IRI) and other clinical-microbiological information (e.g., for differential diagnosis of INF) in routine multidisciplinary team discussions.

Moreover, based on our findings, a cross-evaluation of different cell immunostainings with different sensitivity and specificity (high sensitivity in macrophages and high specificity in endothelial cells) could lead to a more likely conclusive interpretation of AMR. It is well known that no single antibody is 100% sensitive and specific in pathology practice. A final diagnosis is usually made after considering several aspects, including histology, the use of ancillary tools and multidisciplinary team discussion. To evaluate AMR in pathology, biomarkers that are easy to approach and assess in routine diagnostic practice should be used. In our study, C4d immunoassaying had low sensitivity in lung AMR, being positive in only 21% of cases, which resulted in a downgraded diagnosis level. If we had used p-S6RP in the diagnostic algorithm, 16 probable AMR cases would have been upgraded to definite. Optimal treatment for AMR is still poorly defined, particularly for probable and possible cases. A downgraded diagnosis could have a significant impact on patient treatment planning and management.

C4d negativity could be explained by a relative lack of sensitivity of the staining in protocol biopsy specimens or by the involvement of antibodies with a too low affinity or unable to fix complement.[26] Moreover, several data, mainly coming from experimental studies, also support the concept of a complement-independent pathway, thus arguing that lack of C4d deposition does not exclude AMR.[27,28] The evidence of negative C4d AMR is progressively increasing today, even in organs where this marker has been considered very sensitive. Indeed C4d-negative AMR has been recognized in kidney transplantation and has been integrated since 2011 in the Banff schema.[29] The occurrence of microcirculation injury without C4d deposition in the kidney as well as in cardiac post-transplant biopsies in the latter, sometimes associated with activated intravascular macrophages (CD68 positive) are now considered to be highly suggestive of AMR.[1,21,30] Thus, it is mandatory to also search in the lung for other biomarkers that can increase the sensitivity and help in the diagnostic algorithm, and so should be for p-S6RP immunostaining.

While the detection of phosphorylated (p)-mTOR and its downstream S6RP signals seem to be useful diagnostic biomarkers of AMR in several solid organ transplants its use as a target for mTOR treatments is currently under debate.[10,31]

Larger studies are needed to confirm our findings and to determine whether p-S6RP could be used to assess the value of treatment efficacy in late AMR in clinical trials.

Limitations and strengths

One of the most important strengths of this study that tries to address key knowledge gap in lung transplant AMR biomarkers is the multicenter design. Indeed, a substantial number of patients from different centers belonging to the case and control groups. Our study is a retrospective analysis, and a major limitation is the availability of complete clinical data. Although treatment regimens, pulmonary function data, and progression to CLAD are available in some cases, they are incomplete in others. To minimize confounding factors such as induction therapy and AMR treatment, we established strict inclusion criteria and excluded patients with preformed DSA, only including biopsies obtained contemporaneously with the first discovery of de novo DSA. However, the study only includes clinical AMR, underrepresenting the value of this biomarker in subclinical cases, which are even more challenging to diagnose.

Disclosure statement

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

All authors contributed to the acquisition of the clinical or biological data for this study; FL, GAF, FC contributed to the study design; FL, LV, FC contributed to the development of the analysis plan; FL, FP, PD, MI, TP, KW, MPC, RAS, GAF and FC performed the pathological analyses of cases; JLP, MP, SH, EC and FR performed the clinical/immunological study; FL, FP, PD, MI, TP, KW, MPC, RAS, GAF, FC, MG, DN, AMF, AR, EC, DJL and AR reviewed and discussed the cases; LV performed the analyses. All authors contributed to the interpretation of the data. FL and FC drafted the manuscript. All authors critically revised the manuscript and approved the final version for submission.

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Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.healun.2023.10.002.

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