Sheddindemg light on the problematic nature of C4d in lung transplantation –a quantitative analysis of bronchoalveolar lavage fluid

Tobias Heigl, Berta Saez-Gimenez, Anke Van Herck, Janne Kaes, Annelore Sacreas, Hanne Beeckmans, Gene P.L. Ambrocio, Hanneke Kwakkel-Van Erp, Sofie Ordies, Arno Vanstapel, Stijn E. Verleden, Arne P. Neyrinck, Laurens J. Ceulemans, Dirk E. Van Raemdonck, Erik Verbeken, Geert M. Verleden, Robin Vos, Bart M. Vanaudenaerde

PII: S0966-3274(20)30119-2

DOI: <https://doi.org/10.1016/j.trim.2020.101352>

Reference: TRIM 101352

To appear in: *Transplant Immunology*

Received date: 10 September 2020

Revised date: 7 November 2020

Accepted date: 7 November 2020

Please cite this article as: T. Heigl, B. Saez-Gimenez, A. Van Herck, et al., Sheddindemg light on the problematic nature of C4d in lung transplantation –a quantitative analysis of bronchoalveolar lavage fluid, *Transplant Immunology* (2020), <https://doi.org/10.1016/j.trim.2020.101352>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Sheddindemg Light on the Problematic Nature of C4d in Lung Transplantation –
A Quantitative Analysis of Bronchoalveolar Lavage Fluid.**

Tobias Heigl^{1*}, Berta Saez-Gimenez^{2*}, Anke Van Herck¹, Janne Kaes¹, Annelore Sacreas¹,
Hanne Beeckmans¹, Gene PL Ambrocio¹, Hanneke Kwakkel-Van Erp^{1,3}, Sofie Ordies¹, Arno
Vanstapel¹, Stijn E Verleden¹, Arne P. Neyrinck¹, Laurens J. Ceulemans¹, Dirk E Van
Raemdonck¹, Erik Verbeken⁴, Geert M Verleden¹, Robin Vos¹, Bart M Vanaudenaerde¹

¹ Department of Chronic Diseases, Metabolism & Ageing (CHROMETA); Laboratory of Respiratory Diseases & Thoracic Surgery (BREATHE), UZ/KU Leuven, Leuven, Belgium

²Department of Pneumology, University Hospital Vall d'Hebron, Barcelona, Spain

³Department of Pneumology, University of Antwerp, Antwerp, Belgium

⁴Translational Cell and Tissue Research, KU Leuven and UZ Gasthuisberg, Leuven, Belgium

* Contributed equally to the work

Keywords: Complement factor C4d, Lung Transplantation, Broncho-alveolar lavage, Antibody Mediated Rejection, Chronic Lung Allograft Dysfunction.

Corresponding Author: Prof. Dr. Bart Vanaudenaerde

Dept. of Chronic Diseases, Metabolism and Ageing (CHROMETA),

Lab of Respiratory Diseases and Thoracic Surgery (BREATHE),

Lung Transplant Unit

KU Leuven and UZ Leuven

Herestraat 49, B-3000 Leuven, Belgium

Tel: + 32 16 330194 Fax: + 32 16 346803

E-mail: bart.vanaudenaerde@kuleuven.be

ABBREVIATIONS

AMR: Antibody mediated rejection

AR: Acute rejection

BAL: Broncho-alveolar lavage

BOS: Bronchiolitis obliterans syndrome

C4d: Complement factor 4d

CLAD: Chronic lung allograft dysfunction

CRP: C-reactive protein

HLA: Human leukocyte antigen

INF: Infection

LB: Lymphocytic bronchiolitis

MAC: Membrane attack complex

RAS: Restrictive allograft syndrome

ABSTRACT

In recent years, the utility of vascular complement factor 4d (C4d) deposition as diagnostic tool for antibody mediated rejection (AMR) after lung transplantation, has become a controversial issue. We aimed to pinpoint the problematic nature of C4d as biomarker with a simple experiment.

We quantified C4d in broncho-alveolar lavage (BAL) of lung transplant patients with diverse post-transplant complications in 3 different settings of clinically clear cases of: 1/ chronic lung allograft dysfunction (CLAD); 2/ acute complications acute rejection (AR), lymphocytic bronchiolitis (LB), antibody-mediated rejection (AMR) and respiratory infection (INF); 3/ patients with parallel C4d immunostaining and Anti-HLA. All groups were compared to BAL of stable patients. C4d was measured via standard ELISA.

C4d was increased in CLAD, predominantly in RAS ($p=0.0026$) but not in BOS ($p=0.89$). C4d was increased in all acute events, AR ($p=0.0025$), LB ($p<0.0001$), AMR ($p=0.0034$), infections ($p<0.0001$). In patients with parallel C4d immunostaining and serum HLA antibodies, C4d was increased in C4d-/HLA- ($p=0.0011$); C4d-/HLA+ ($p=0.013$); HLA+/C4d+ ($p=0.0081$). A correlation of systemic C-reactive protein (CRP) with C4d was found in all patients ($r=0.43$; $p<0.0001$). We hypothesize that free C4d in BAL may only be representative of a general immune response in the transplanted lung.

INTRODUCTION

Chronic Lung Allograft Dysfunction (CLAD) remains the main cause of death beyond the first year after lung transplantation. Important risk factors for CLAD are acute cellular rejection (AR), lymphocytic bronchiolitis (LB), antibody mediated rejection (AMR) and infections (INF) (1–3). Early diagnosis and understanding the pathomechanisms, of the, aforementioned complications, in both their similarities and diversities are key elements in improving post lung transplant (LTx) outcomes. This is reflected by efforts to identify clear-cut biomarkers for post-transplant complications (4,5). One of the most elusive complications is antibody-mediated rejection (AMR). AMR is recognized across all forms of solid organ transplantation, in which alloantibodies mediate a substantial proportion of graft-rejection episodes, contributing to both early and late graft loss (6). As the pathophysiology of AMR is poorly understood, proper diagnostic and therapeutic tools are currently lacking (7,8). Feucht et al. however, showed that C4d, a split product of C4b, deposition in small blood vessels on kidney transplant biopsies could be a biomarker for AMR (9).

The complement system was discovered by the Belgian noble prize winner Jules Bordet and is described as a cascade of factors complementing the immune system (from which its name is derived) in a non-specific way. It facilitates the ability of antibodies and phagocytic cells to clear microbes and damaged or non-self-cells from an organism, promote inflammation, and attack the cell membrane of pathogens (10).

The involvement of the complement system and its fragments in transplantation was first identified in hyperacute rejection in 1988 (11) and only later on in acute and chronic rejection in kidney transplantation (9). Also the heart transplant community adopted C4d as histological marker for AMR and concluded that C4d staining may help to identify patients with a combination of acute cellular and humoral rejection (12). C4d immunostaining that identified the stable covalent binding specific to the capillary endothelium was put forward as a specific (bio)marker for AMR. It was proposed that this strong thioester binding group prevents

shedding off from endothelial cells. (13). C4d staining as potential biomarker of AMR was adapted from other solid organ transplantations by the lung and heart transplant community and is now part of the ISHLT guidelines on AMR without further organ specific validation (14,15). However, right from the start C4d staining as potential read-out for AMR remained troublesome in lung transplantation, due to its poor reproducibility (high background and focal staining)(16), difficult interpretation (emergence of C4d negative AMR as a new entity)(17) and overlap with other complications.

In the meantime, C4d measurement by simple ELISA methodology within Broncho-Alveolar Lavage fluid (BAL) was put forward and linked to restrictive allograft dysfunction (RAS) (18,19).

Due to the lasting controversies, we aimed to pinpoint the problematic nature of C4d as biomarker with a simple experiment. We assessed patient groups, based on guidelines for diagnosis of post-transplant complications (CLAD, lymphocytic bronchiolitis, acute cellular rejection, antibody mediated rejection and infections) and whether C4d can aid in differential diagnosis of AMR. To this end we used broncho-alveolar lavage fluid from our biobank, performed a conventional quantitative ELISA and compared all patient groups to stable LTx patients

METHODS

C4d ELISA was analyzed in BAL within 3 different experiments including a selection of patients with CLAD, both BOS and RAS (experiment 1; CLAD subdivision); patients with clinical diagnosed acute complications, namely AR, LB, AMR and INF (experiment 2; acute clinical complication subdivision); and finally, patients in whom a parallel C4d immunostaining on transbronchial biopsy and serum HLA quantification was also performed for clinical concerns (experiment 3; HLA and C4d IHC based subdivision). Clinically stable lung transplant patients were used as control group within each experiment. We

retrospectively selected BAL samples (from our institution's biobank (S51577) including patients informed consent) and reviewed patient characteristics, including gender, age, type of lung transplantation and underlying disease. All patients received induction therapy and afterwards, triple immunosuppression treatment with calcineurin inhibitor, methylprednisolone and mycophenolate mofetil or azathioprine.

Patients/BAL samples were selected to create clear groups based on standard and obvious clinical, immunopathological and radiological characteristics. Per experiment, a patient could only be included with 1 sample in one group. Samples of patients who had, at that time of assessment, multiple complication (e.g. infection and RAS) were excluded.

The BOS (n=14) and RAS (n=16) subgroups were selected based on retrospective diagnosis of CLAD (BOS and RAS) based on histopathology, spirometry and radiology (CT scan) data (20). A BAL sample at time of diagnosis was used. AR (n=5) and LB (n=9) groups were selected based on histopathological criteria (21). AMR (n=5) includes definite and probable AMR according to the ISHLT consensus (14). Respiratory infection (INF)(n=14) was defined by clinical symptoms of infection such as fever, dyspnea with an elevated C reactive protein (CRP) with/without chest X-ray changes and positive cultures (blood/sputum), with need for antibiotics resulting in a FEV₁ improvement.

For the last experiment, we assessed all C4d immunostainings (performed for diagnostical assistance) on transbronchial biopsies with concurrent serum HLA quantification and BAL availability. The reasons for this extra clinical examination included suspicion of infection, acute or chronic rejection, AMR, infection or lung functional decline in general. These samples were grouped as follow: C4d-/HLA- (n=13), C4d-/HLA+ (n=9), C4d+/HLA- (n=2) and C4d+/HLA+ (n=5). All groups were compared to clinically stable lung transplantation patients, where a parallel C4d staining was not performed as no indication for a complication was present.

LTx recipients are routinely screened for Anti-HLA antibodies (HLA Ab) during the first year after transplantation, at diagnosis or suspicion of CLAD, AMR, or a yet unknown post-transplant complication. Anti-HLA antibodies were detected by Luminex technology according to the institutions' protocol and were classified as either positive or negative. As previously published, a HLA screening result was considered negative if MFI <500 and positive if MFI $\geq$ 500 (22,23).

BAL was performed with 2 aliquots of sterile saline (50 mL) of which the returned fractions were pooled and processed for cell counting as previously described (18). C4d deposition on transbronchial biopsies was assessed by immunohistochemistry at our routine clinical pathology laboratory. In BAL, C4d was measured via immunoassay according to the manufacturer's instructions (Quidel, San Diego, CA). Undiluted BAL samples were analyzed, expressed in ng/ml and with a detection C4d limit of 1ng/ml. If under the detection limit, half of the detection limit was used for further statistical analysis.

Qualitative variables are expressed as absolute numbers and percentages. Normally distributed quantitative variables are expressed as mean and standard deviation; non-normally distributed variables are expressed as median and interquartile range (IQR). Demographic and clinical variables of patients were compared using the chi-square test for qualitative variables. Normally distributed quantitative variables were compared using one-way ANOVA test; non-normally distributed quantitative variables were compared using Mann-Whitney test and Kruskal-Wallis test. Data were analyzed using Graph Pad prism 8.0 software (San Diego, CA, USA).

RESULTS

The levels of C4d in BAL of all patients range from 0 to 407 ng/mL, with a median of 77 ng/mL (IQR 10-154). Demographic characteristics of experiment 1 (CLAD) are summarized in table 1.

C4d in BAL (figure 1A) was significantly increased in patients with RAS (146 ± 107 ng/mL) versus stable patients (41 ± 56 ng/mL; $p=0.0026$) and patients with BOS (27 ± 35 ng/mL; $p=0.007$) patients. No significant difference was seen between stable and patients BOS C4d levels in BAL ($p=0.89$).

Patient characteristics of experiment 2 (acute complications) are described in table 2. As expected, DSA were more frequently found in the AMR. Due to the cross-sectional nature of the study there were differences regarding time from lung transplantation to BAL sampling across the groups: it was longer in the stable, AMR and LB groups compared to AR and infection. Follow-up time was shorter in the AMR group. BAL C4d (figure 1B) was significantly increased in all groups. When compared to the stable group, the highest C4d mean level was seen in the INF (197 ± 144 ng/mL; $p<0.0001$) group, followed by the AMR (167 ± 132 ng/mL; $p=0.0034$), IR (95 ± 40 ng/mL; $p<0.0001$) and AR (87 ± 78 ng/mL; $p=0.0025$) groups.

Regarding experiment 3 (subdivision based on C4d IHC and serum HLA present), there were no significant differences in clinical characteristics between groups (table 3). BAL C4d levels (figure 1C) were significantly increased in C4d-/HLA- (141 ± 103 ng/mL; $p=0.00011$); C4d-/HLA+ (108 ± 85 ng/mL; $p=0.013$) and C4d+/HLA+ (167 ± 132 ng/mL; $p=0.0004$) patients versus stable (39 ± 47 ng/mL) patients, whereas no significant difference was seen in C4d+/HLA- (123 ± 79 ng/mL; $p=0.30$).

Finally, there was a significant correlation between (log transformed to research normality testing) CRP and C4d in BAL of all patients (figure 1D) ($r=0.49$; $p<0.0001$).

DISCUSSION

This study investigated how free C4d in BAL relates to post transplant complications. C4d was increased in all groups and did not show specificity towards a certain complication, also not AMR.

The specificity was investigated in two different settings. Experiment 1, focusing on the chronic complication CLAD, showed increased BAL C4d in RAS (but not BOS) compared to stable patients. This increase in C4d can be linked to the humoral immunity involved in the RAS phenotype of CLAD, as previously suggested with the presence of DSA and immunoglobulins (18). It also opens up new perspectives as in patients with increased BAL C4d where all acute complications were excluded; one might suspect an onset of RAS. However, this is beyond the scope of this study

In contrast, experiment 2 which focuses on clinically diagnosed acute post-transplant complications had increased BAL C4d in all entities (infection, LB, AMR and AR) compared to stable patients. These observations are in line with the study of Girnita et al. (19) which showed similar results regarding increased BAL C4d in patients infections. One could argue that C4d in BAL is only a read out for the airway compartment of the lung and not the vasculature, explaining the non-specificity of C4d in BAL. However, previous studies have shown capillary deposition of C4d in the vessels of patients with post lung transplant infections and PGD (19,24), which points towards the non-specific nature of C4d itself. The observed moderate correlation between BAL C4d and systemic CRP is another indication of non-specificity, reinforcing the idea put forward by others (15) that the classic pathway of the complement system can also be activated by CRP and surface components of gram-positive bacteria. Another explanation would be that C4d, as CRP, represents a general immune response, which would seem logical due to the unspecific nature of the complement system. Moreover, C4d in BAL also correlated with neutrophilia and IL-6 and IL-8 within the BAL

confirming the non-specificity of the increase over the different complication ($r^2=0.084$; $r^2=0.074$; $r^2=0.051$ respectively; data non-shown). Simply dividing samples based on negativity/positivity of C4d staining and HLA quantification (experiment 3) showed no difference between the subgroups. All were increased when compared to control samples. Although the low n-values do not allow for a final verdict, experiment 3 further corroborates the issue of specificity and low sensitivity of C4d. Additional survival analysis of the subgroup of BAL samples of the 4 diagnostical groups where both a C4d immunostaining and serum Anti-HLA quantification was performed, divided based on C4d >100 ng/ml versus C4d <100 ng/ml did not show a higher CLAD development or mortality ($p=0.78$ and 0.17 ; data not shown).

The existence of “C4d negative AMR” has been described in different studies in lung transplantation (25,26), and has recently been acknowledged in the ISHLT consensus (14), which recognizes the diagnosis of AMR with or without C4d deposition. In contrast to our results, Garnita et al. (19) described the highest levels of C4d in patients with both anti-HLA Ab and positive C4d staining followed by patients with anti-HLA Ab but negative C4d staining, and the lowest level in patients where both were negative.

Clear limitations of this study are its small sample size and cross-sectional nature and it should therefore be seen as hypothesis generating only.

We hypothesize that free C4d in BAL is a left-over product generated by a general immune response, which cannot be traced back to a specific post-LTx complication. We do not aim for a big statement but a critical reappraisal of use of C4d.

ACKNOWLEDGMENT

Funding: BV is supported by the KU Leuven (C16/19/005). GMV and DEVR are supported by a grant from the Broere foundation. RV is a Senior Clinical Research Fellow of the

Research Foundation Flanders (FWO; 12G8715N). SEV is a Post-doctoral Research Fellow of the Research Foundation Flanders (FWO 12G8715N and 1500617N) Belgium. AN is supported by the KU Leuven (C24/18/073). JK and AV are junior research fellow of the Research Foundation Flanders (1198920N and 1102020N). GPA is supported by the European Respiratory Society - ERS Clinical Training Fellowship.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose.

FIGURE LEGENDS

Figure 1: C4d levels in Broncho-alveolar lavage fluid in different patient groups. (A) BAL C4d in CLAD patients (BOS and RAS) vs. control; (B) BAL C4d in acute clinical complications vs. control; (C) BAL C4d in patients grouped based on lung transbronchial biopsy C4d staining and parallel HLA Ab measurement. (D) Correlation between BAL C4d and CRP in serum. The C4d and CRP values were log transformed.

REFERENCES

1. Verleden GM, Vos R, Vanaudenaerde B, Dupont L, Yserbyt J, Van Raemdonck D, et al. Current views on chronic rejection after lung transplantation. *Transpl Int*. 2015 Oct;28(10):1131–9.
2. Verleden SE, Ruttens D, Vandermeulen E, Vaneylen A, Dupont LJ, Van Raemdonck DE, et al. Bronchiolitis obliterans syndrome and restrictive allograft syndrome: do risk factors differ? *Transplantation*. 2013 May 15;95(9):1167–72.
3. Meyer KC, Raghu G, Verleden GM, Corris PA, Aurora P, Wilson KC, et al. An international ISHLT/ATS/ERS clinical practice guideline: diagnosis and management of bronchiolitis obliterans syndrome. *Eur Respir J*. 2014 Dec;44(6):1479–503.
4. Speck NE, Probst-Müller E, Haile SR, Benden C, Köhler M, Huber LC, et al. Bronchoalveolar lavage cytokines are of minor value to diagnose complications following lung transplantation. *Cytokine*. 2019 Aug 7;125:154794.
5. Parulekar AD, Kao CC. Detection, classification, and management of rejection after lung transplantation. *J Thorac Dis*. 2019 Sep;11(Suppl 14):S1732–9.
6. Loupy A, Lefaucheur C. Antibody-Mediated Rejection of Solid-Organ Allografts. *N Engl J Med*. 2018 20;379(12):1150–60.
7. Magro CM, Pope Harman A, Klinger D, Cruz C, Adams P, Waldman J, et al. Use of C4d as a diagnostic adjunct in lung allograft biopsies. *Am J Transplant*. 2003 Sep;3(9):1143–54.
8. Wallace WD, Reed EF, Ross D, Laksman CR, Fishbein MC. C4d staining of pulmonary allograft biopsies: an immunoperoxidase study. *J Heart Lung Transplant*. 2005 Oct;24(10):1565–70.
9. Feucht HE, Felber E, Göbel MJ, Hillebrand G, Nattermann U, Brockmeyer C, et al. Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. *Clin Exp Immunol*. 1991 Dec;86(3):464–70.
10. Ricklin D, Hajishensallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol*. 2010 Sep;11(9):785–97.
11. Banner B, Makowka L, Demetris J, Tzakis A, Griffin M, Starzl TE. Hyperacute Rejection of the Kidney in Patients With a Negative Crossmatch. *Transplant Proc*. 1988 Feb;20(1 Suppl 1):453–9.
12. Behr TM, Feucht HE, Richter K, Reiter C, Spes CH, Pongratz D, et al. Detection of humoral rejection in human cardiac allografts by assessing the capillary deposition of complement fragment C4d in endomyocardial biopsies. *J Heart Lung Transplant*. 1999 Sep;18(9):904–12.
13. Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N, et al. Complement Activation in Acute Humoral Renal Allograft Rejection: Diagnostic Significance of C4d Deposits in Peritubular Capillaries. *JASN*. 1999 Oct 1;10(10):2208–14.

14. Levine DJ, Glanville AR, Aboyoun C, Belperio J, Benden C, Berry GJ, et al. Antibody-mediated rejection of the lung: A consensus report of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant*. 2016 Apr;35(4):397–406.
15. Westall GP, Snell GI. Antibody-mediated rejection in lung transplantation: fable, spin, or fact? *Transplantation*. 2014 Nov 15;98(9):927–30.
16. Roden AC, Maleszewski JJ, Yi ES, Jenkins SM, Gandhi MJ, Scott JP, et al. Reproducibility of Complement 4d deposition by immunofluorescence and immunohistochemistry in lung allograft biopsies. *The Journal of Heart and Lung Transplantation*. 2014 Dec;33(12):1223–32.
17. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, et al. Banff 2013 Meeting Report: Inclusion of C4d-Negative Antibody-Mediated Rejection and Antibody-Associated Arterial Lesions: Banff 2013 Meeting Report. *American Journal of Transplantation*. 2014 Feb;14(2):272–83.
18. Vandermeulen E, Verleden SE, Bellon H, Ruttens D, Lammertyn E, Claes S, et al. Humoral immunity in phenotypes of chronic lung allograft dysfunction: A broncho-alveolar lavage fluid analysis. *Transpl Immunol*. 2016;38:27–32.
19. Girit AL, Lee TM, McCurry KR, Baldwin VM, Yousem SA, Detrick B, et al. Anti-human leukocyte antigen antibodies, vascular C4d deposition and increased soluble c4d in broncho-alveolar lavage of lung allografts. *Transplantation*. 2008 Jul 27;86(2):342–7.
20. Verleden GM, Raghu G, Meyer KC, Glanville AR, Corris P. A new classification system for chronic lung allograft dysfunction. *J Heart Lung Transplant*. 2014 Feb;33(2):127–33.
21. Stewart S, Fishbein MC, Snell GI, Berry GJ, Boehler A, Burke MM, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. *J Heart Lung Transplant*. 2007 Dec;26(12):1229–42.
22. Verleden SE, Vanaudenaerde BM, Emonds M-P, Van Raemdonck DE, Neyrinck AP, Verleden GM, et al. Donor specific and -nonspecific HLA antibodies and outcome post lung transplantation. *European Respiratory Journal* [Internet]. 2017 Nov 1 [cited 2020 Jul 14];50(5). Available from: <https://erj.ersjournals.com/content/50/5/1701248>
23. Sacreas A, Taupin J-L, Emonds M-P, Daniëls L, Raemdonck DEV, Vos R, et al. Intragraft donor-specific anti-HLA antibodies in phenotypes of chronic lung allograft dysfunction. *European Respiratory Journal* [Internet]. 2019 Jan 1 [cited 2020 Jul 14]; Available from: <https://erj.ersjournals.com/content/early/2019/07/31/13993003.00847-2019>
24. Westall GP, Snell GI, McLean C, Kotsimbos T, Williams T, Magro C. C3d and C4d deposition early after lung transplantation. *J Heart Lung Transplant*. 2008 Jul;27(7):722–8.
25. Otani S, Davis AK, Cantwell L, Ivulich S, Pham A, Paraskeva MA, et al. Evolving experience of treating antibody-mediated rejection following lung transplantation. *Transpl Immunol*. 2014 Aug;31(2):75–80.

26. Aguilar PR, Carpenter D, Ritter J, Yusef RD, Witt CA, Byers DE, et al. The role of C4d deposition in the diagnosis of antibody-mediated rejection after lung transplantation. *Am J Transplant*. 2018 Apr;18(4):936–44.

Table 1: Patient characteristics in CLAD (experiment 1).

	Control n= 13	BOS n= 14	RAS n= 16	P
Age, median (IQR)	51 (36-63)	35 (29-52)	39 (23-52)	0.14
Gender: Male, n (%)	4 (31)	8 (57)	10 (63)	0.20
Diagnosis, n (%)				0.30
COPD	5 (39)	3 (21)	5 (31)	
ILD	2 (15)	1 (7)	2 (13)	
CF	4 (31)	1 (7)	3 (19)	
Other	2 (15)	5 (64)	6 (38)	
Type of LTx, n (%)				0.17
SSLT	13 (100)	13 (93)	14 (88)	
SLT	0	1 (7)	0	
HLT	0	0	2 (13)	
DSA, n(%)	0	0	3(19)	0.027
Time between LTx and BAL (months), median (IQR)	24 (24-25)	28 (13-61)	28 (12-55)	0.99
Time to CLAD (months), median (IQR)	/	26 (15-61)	25 (11-56)	0.95
Time follow up (months), median (IQR)	77 (76-84)	47 (26-72)	42 (18-96)	0.035

List of abbreviations: COPD=chronic obstructive pulmonary disease; ILD=Interstitial Lung Disease, CF=Cystic Fibrosis, LTx=Lung transplantation; SSLT=Single sequential lung transplantation; SLT=Single Lung Transplantation; HLT=Heart-Lung Transplantation; DSA=Donor Specific Antibodies; CLAD= Chronic Lung Allograft Dysfunction.

Table 2: Patient characteristics in acute complications (experiment 2).

	Control n= 12	AR n= 10	INF n= 14	AMR n=5	LB n= 9	P
Age, median (IQR)	51 (31-61)	57 (46-62)	56 (50-60)	45 (25-57)	54 (39-63)	0.64
Gender: Male, n (%)	3 (25)	4 (40)	8 (57)	2 (40)	4 (44)	0.59
Diagnosis, n (%)						0.26
COPD	5 (42)	4 (40)	6 (43)	1 (20)	6 (67)	
ILD	2 (17)	5 (50)	4 (29)	3 (60)	0	
CF	4 (33)	1 (10)	1 (7)	1 (20)	2 (22)	
Others	1 (8)	0	3 (21)	0	1 (11)	
Type of LTx, n (%)						0.27
SSLT	12 (100)	8 (80)	12 (93)	5 (100)	9 (100)	
SLT	0	2 (20)	1 (7)	0	0	
HLT	0	0	0	0	0	
DSA, n (%)	0	2 (20)	1 (8) ¹	5 (100)	1 (11)	<0.0001
Time between LTx and BAL (months), median (IQR)	25 (24-25)	5 (3-23)	6 (3-20)	26 (9-46)	25 (20-69)	0.16
Time follow up (months), median (IQR)	77 (76-79)	100 (64-115)	67 (13-7)	55 (48-83)	103 (85-142)	0.011
Concomitant infection, n (%) [*]	0	0	14 (100)	0	1 (11)	<0.0001

List of abbreviations: COPD=chronic obstructive pulmonary disease; ILD=Interstitial Lung Disease; CF= Cystic Fibrosis, LTx= Lung transplantation; SSLT= Single sequential lung transplantation; SLT= Single Lung Transplantation; HLT= Heart-Lung Transplantation; DSA= Donor Specific Antibodies. AR= acute rejection; INF= infection; AMR antibody mediated rejection; LB lymphoid bronchiolitis. ^{*} Concomitant infection meaning lower respiratory infection requiring treatment.

Table 3: Patient characteristics in groups divided based on C4d IHC and serum HLA positivity (experiment 3).

	Control n= 12	C4d-/HLA- n= 13	C4d-/HLA+ n= 9	C4d+/HLA- n= 2	C4d+/HLA+ n= 5	P
Age, median (IQR)	54 (42-62)	56 (36-62)	36 (28-50)	56 (54-58)	52 (31-60)	0.30
Gender: Male, n (%)	4 (33)	3 (23)	2 (33)	0 (0)	2 (40)	0.13
Diagnosis, n (%)						0.44
COPD	5 (42)	7 (54)	1 (11)	1 (50)	2 (40)	
ILD	2 (17)	2 (15)	3 (33)	0	2 (40)	
CF	3 (25)	0	2 (22)	0	1 (20)	
Others	2 (17)	4 (31)	3 (33)	1 (50)	0	
Type of LTx, n (%)						0.60
SSLT	12 (100)	11 (85)	7 (77)	2 (100)	5 (100)	
HLT	0	2 (15)	2 (22)	0	0	
DSA, n (%)	0	0	2 (100)	0	5 (100)	<0.0001
Time between LTx and BAL (months), median (IQR)	24 (23-24)	55 (11-95)	24 (1-59)	47 (1-93)	25 (9-44)	0.12
Time follow up (months), median (IQR)	76 (74-77)	82 (45-117)	49 (31-90)	78 (58-98)	54 (47-81)	0.59
Concomitant infection, n(%)*	0	2 (23)	2 (22)	0	0	0.46

List of abbreviations: ILD=Interstitial Lung Disease, CF= Cystic Fibrosis, LT= Lung transplantation; SSLT= Single sequential lung transplantation; SLT= Single Lung Transplantation; HLT= Heart-Lung Transplantation; DSA= Donor Specific Antibodies.

* Concomitant infection meaning lower respiratory infection requiring treatment.

Figure 1.