Review

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Biomarkers for Chronic Lung Allograft Dysfunction: Ready for Prime Time?

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Abstract. Chronic lung allograft dysfunction (CLAD) remains a major hurdle impairing lung transplant outcome. Parallel to the better clinical identification and characterization of CLAD and CLAD phenotypes, there is an increasing urge to find adequate biomarkers that could assist in the earlier detection and differential diagnosis of CLAD phenotypes, as well as disease prognostication. The current status and state-of-the-art of biomarker research in CLAD will be discussed with a particular focus on radiological biomarkers or biomarkers found in peripheral tissue, bronchoalveolar lavage, and circulating blood, in which significant progress has been made over the last years. Ultimately, although a growing number of biomarkers are currently being embedded in the follow-up of lung transplant patients, it is clear that one size does not fit all. The future of biomarker research probably lies in the rigorous combination of clinical information with findings in tissue, bronchoalveolar lavage, or blood. Only by doing so, the ultimate goal of biomarker research can be achieved, which is the earlier identification of CLAD before its clinical manifestation. This is desperately needed to improve the prognosis of patients with CLAD after lung transplantation.

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INTRODUCTION

Lung transplantation is the last-resort option for well-selected patients suffering from end-stage chronic diseases such as chronic obstructive pulmonary disease, interstitial lung disease, cystic fibrosis, and others. Although short-term survival has gradually improved to a 1-y survival of about 85%, the median long-term survival of 6 y remains lower compared with other solid organ transplants, where limited additional survival benefit has been achieved in the most recent years.[1] This is mainly explained by the development of chronic rejection, clinically defined as chronic lung allograft dysfunction (CLAD), which is one of the main reasons for long-term morbidity and mortality. The lung indeed has the highest rejection rates among all solid organ transplantations and limited therapeutic options exist that slow down or reverse disease progression.

In the current era of lung transplantation, a lot of research has been dedicated to adequate defining and subtyping of CLAD.[2] CLAD is defined as a persistent decline (≥20%) in forced expiratory volume in 1 s (FEV_1) from the baseline. This baseline value is calculated as the mean of the best 2 postoperative FEV_1 measurements. It is important to realize that also other reasons can cause a similar decline in FEV_1, which are not considered as a manifestation of CLAD, including physiological aging, surgical factors, mechanical factors, infection, lung neoplasms, recurrence of native lung disease, and drug-induced toxicity among others. Based on the evidence of obstruction or restriction on pulmonary function and radiology, additional CLAD phenotypes can be identified, including bronchiolitis obliterans syndrome (BOS), restrictive allograft syndrome (RAS), mixed and undefined (Table 1). The most common phenotype (approximately 60% of CLAD patients[3]) is BOS, characterized by physiologic obstruction (FEV_1/forced vital capacity [FVC] <0.70), absence of restriction (no decline in total lung capacity [TLC] >10%), and no signs of persistent radiologic opacities, which is associated with a better survival post CLAD diagnosis. RAS is less frequently found (approximately 10%–30%[3]) and is defined by the absence of obstruction and presence

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TABLE 1.

Classification of main CLAD phenotypes as recognized by the ISHLT consensus document[2]

CLAD phenotype	Obstruction	Restriction	RLO	Incidence	Prognosis
BOS	+	−	−	50%–70%	3–5 y
RAS	−	+	+	10%–25%	0.5–1.5 y
Mixed	+	+	+	10%–20%	Depending on RLO
Undefined	+	−	+	10%–15%	Depending on RLO
	+	+	−		

BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; ISHLT, International Society for Heart and Lung Transplantation; RAS, restrictive allograft syndrome; RLO, radiologic lung opacities.

of restrictive features (FEV_1/FVC ≥0.70 and TLC decline ≥10%) with the presence of concurrent persistent radiological opacities. Diagnosis of a RAS phenotype of CLAD has been unequivocally associated with a worse survival postdiagnosis compared with the more common BOS phenotype both in single- and double-lung transplant patients.[2,4-6] The term mixed was introduced for those patients that transition during their follow-up from a typical BOS to a more RAS-like phenotype (FEV_1/FVC <0.70, TLC decline ≥10%, and persistent radiological opacities). Approximately 10% of patients develop such a phenotype transition,[7] and as soon as persistent radiological opacities develop, survival is impaired[8] and in line with the survival of an initial RAS diagnosis. There is more uncertainty about the exact manifestation and etiology of the undefined phenotype, defined as presence of obstructive lung physiology with concomitant radiological opacities or presence of restriction without radiological opacities. However, as soon as persistent radiological opacities develop, survival is seriously impaired to 1 y following the appearance of radiological opacities.[3] An unclassified phenotype has also been proposed for patients fitting none of the previous categories, but their significance needs to be further validated and is an important target for future research.[3]

Although the radiologic and physiologic criteria are used to differentially diagnose the different CLAD phenotypes, there are also major differences in their histopathologic presentation.[9] BOS is typically characterized by obliterative bronchiolitis (OB), a pathologic scarring of the small airways leading to irreversible airway obstruction. RAS, on the other hand, shows an initial pattern of alveolar fibroelastosis, a pattern of predominant subpleural and paraseptal collagenous obliteration of the alveoli with elastosis, with (myo-)fibroblast accumulation and fibrosis,[9] where also concomitant airway obstruction in the form of OB is often observed.

Given the important prognostic implications of developing CLAD but also that it remains difficult to differentially diagnose the CLAD phenotypes, it is important to search for surrogate markers that can assist in subtyping CLAD. These surrogate markers can stem from a lot of different sources including in-depth evaluation of the patients (ie, imaging, pulmonary function) but also from biological samples that can be obtained from the patients including bronchoalveolar lavage (BAL), transbronchial biopsy, or blood. Interestingly, repeated sampling is customary in a lung transplant setting, which provides the opportunity to predict disease development or progression before the process has been clinically defined. In particular, it is of importance to predict CLAD, CLAD phenotype, or CLAD progression before the actual pulmonary function decline is clinically found, which provides a window of opportunity for early therapeutic intervention or aggressive treatment of obvious risk factors such as antibody-depleting therapies. In this overview, we will provide a comprehensive overview of different biomarkers that have been investigated (summarized in Table 2). Ideally, a good biomarker bears 3 important technical attributes. Firstly, the marker must be present in peripheral tissue and/or fluid. Secondly, it must be easy to detect or quantify in an affordable and robust way. Thirdly, its appearance must be associated as specifically as possible, preferably in a quantifiable way. These biomarkers bear the potential to reflect disease severity and could be used to monitor therapeutic interventions. We are convinced that proper phenotyping of patients in the different CLAD phenotypes is crucial in determining the predictive power of a biomarker.

RADIOLOGIC ASSESSMENT

The recent consensus guidelines from the International Society for Heart and Lung Transplantation have increased the relative weight for radiological assessment in the differential CLAD diagnosis. Typical patterns of fibrosis have been identified in a significant proportion of the CLAD patients where patterns of pleuroparenchymal fibroelastosis, (sub-) pleural thickening, consolidation, ground-glass opacification, and reticulation can be found as a manifestation of persistent radiological opacities typical for RAS patients, in patients that transitioned from BOS to RAS (mixed), and in patients with the undefined phenotype.[87,88] These fibrotic changes are uniformly associated with a poor postdiagnosis outcome.[3] It is also noteworthy that within those patients with persistent opacities, the predominant spatial location within the lung is of prognostic importance with an apical location being associated with a better survival compared with those patients with basal or diffuse predominance of the infiltrates.[37] Given this important diagnostic and prognostic information provided by computed tomography imaging, the interest in radiology as a biomarker for CLAD has been resparked and new tools to assess CTs of patients have been proposed. Artificial intelligence could play an important role in the further establishment of radiology as a biomarker of CLAD. In general, the tools that are currently being considered can be divided in those that assess the airway compartment, the vascular compartment or the interstitium.[89] Firstly, airway obliteration is a pathophysiologic phenomenon found in all CLAD patients also in those patients with a RAS or mixed phenotype.[90,91] Therefore, the focus has been mostly to differentiate BOS and control patients. Measures such as bronchial wall thickness, the wall area ratio and the airway wall area

TABLE 2.

Overview of the different proposed biomarkers with their source, purpose, and their respective associations

Target	Purpose	Type of marker	Association	References
Radiologic assessment				
BOS	Diagnostic	Airway wall and airway parameters	Increased	10-12
BOS	Diagnostic	Vessel cross-sectional area	Decreased	13
CLAD/RAS	Diagnosis + prognostic	Pulmonary vasculature volume	Increased	14
CLAD	Diagnostic	Lung volume and density	Increased and decreased	15-17
BOS	Diagnostic	Software determined air trapping	Increased	18
CLAD/RAS	Diagnostic + prognostic	Small airway and parenchymal disease	Increased	18-21
BOS	Diagnostic	Regional flow-volume loops, ventilation-weighted Fourier (MRI)	Increased	22, 23
RAS	Diagnostic	Apical pleural thickening via lung ultrasound	Increased	24
Tissue markers				
RAS	Risk factor + prognostic	Acute fibrinoid organizing pneumonia	Increased	25, 26
CLAD	Risk factor	Organizing pneumonia and diffuse alveolar damage	Increased	27
CLAD	Risk factor + prognostic	Eosinophils	Increased	28
CLAD	Risk factor	Telomere length	Decreased	29
CLAD	Diagnostic	Molecular signature of wound healing and angiogenesis	Increased	30
CLAD	Prognostic	Molecular signature of T-cell–mediated rejection	Increased	31
CLAD	Risk factor + diagnostic	Profibrotic signature	Increased	32-34
Bronchoalveolar lavage				
CLAD/RAS	Risk factor	Mesenchymal colony forming units	Increased	35, 36
RAS	Risk factor + prognostic	Eosinophils	Increased	37, 38
CLAD	Diagnostic	NK cells	Decreased	39
CLAD	Risk factor	Molecular signature of immune response	Increased	40, 41
CLAD	Risk factor + diagnostic + prognostic	IL-6/IL-6 receptor	Increased	42, 43
RAS	Diagnostic + prognostic	M65 (epithelial cell apoptosis and total cell death)	Increased	44
RAS	Diagnostic	Alveolar alarmins	Increased	45
CLAD	Risk factor	C-X-C Motif Chemokine Receptor 3	Increased	46-48
CLAD	Risk factor	Renin-angiotensin system	Increased	49
RAS	Diagnostic	Humoral immunity	Increased	50
CLAD	Risk factor + prognostic	Bile acids	Increased	51
CLAD	Risk factor + diagnostic + prognostic	Disbalance of the microbiota	Increased	52-56
Circulating markers				
CLAD	Risk factor + prognostic	Donor specific antibodies	Increased	57-61
CLAD	Risk factor	Auto-antibodies	Increased	62-64
CLAD	Risk factor	Donor-derived cell-free DNA	Increased	65-68
CLAD	Risk factor	Acute phase proteins	Increased	69
CLAD	Risk factor + prognostic	Differential cell count (neutrophils, eosinophils, NK cells)	Increased	70-73
CLAD	Risk factor	Regulatory cells	Decreased	74-79
CLAD	Risk factor	Telomere length	Decreased	80-82
CLAD	Risk factor + diagnostic + prognostic	KL-6	Increased	83-86

BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; IL-6, interleukin-6; NK, natural killer; RAS, restrictive allograft syndrome.

ratio to the whole airway have been used to differentiate BOS and controls.[10-12] Secondly, the vasculature could be an important discriminator where, for example, a decrease in vessel cross-sectional area is found in patients with BOS compared with controls.[13] A comprehensive study compared scoring by radiologists with machine learning prediction, and although both radiologist and machine learning scoring were associated with graft failure, the pulmonary vasculature volume, unique to machine learning, was the strongest in phenotyping and prognostication of patients.[14] Lastly, the parenchyma can be comprehensively investigated where a subdivision can be made in those tools that differentiate signs of hyperinflation and air trapping versus those focusing on parenchymal changes related to fibrosis. Simple measures of lung volume and lung density have been used to differentiate the BOS and RAS phenotype with the latter being characterized with a lower lung volume and an increased lung density.[15-17] An increasing number of software packages can also quantitatively assess radiologic phenotypes. For example, BOS patients show a higher degree of air trapping, whereas RAS patients show more signs of interstitial disease.[18] More sophisticated methods leverage available inspiratory and expiratory CTs and perform voxel to voxel comparison to classify every pixel as predominantly functional small airway disease or parenchymal disease. This methodology

has demonstrated that (1) BOS progression is associated with an increase in small airway disease,[19] (2) patients with a restrictive pulmonary function pattern compatible with RAS show more interstitial disease,[20] and (3) important prognostic implication with a 4-fold decrease in CLAD-free survival in those patients with high measures of small airway disease or parenchymal disease.[21] It is noteworthy that also other options are currently being investigated using imaging without exposing the patients to the high dose of radiation of computed tomography. In that aspect, it is of interest that some studies evaluated the use of MRI for CLAD diagnosis where regional flow-volume loops and ventilation-weighted Fourier decomposition detect early-stage CLAD.[22,23] Lastly, lung ultrasound can detect apical pleural thickening and can separate RAS from BOS.[24] Their importance and contribution to CLAD diagnosis and prognosis remain to be investigated and compared with conventional radiologic measures.

TISSUE MARKERS

Although biopsies must be obtained invasively, these provide an undiluted view on the ongoing processes in the allograft. More importantly, biopsies are part of the routine clinical care at regular timepoints of lung transplant patients in most transplant centers, but also when the pulmonary function starts to drop, and are crucial in identifying potential other (identifiable and/or treatable) causes of allograft dysfunction such as infection or acute rejection. Next to in-depth assessment by a pathologist who can assess the presence of distinctive morphological changes, these biopsies can also be assessed for different markers or combination of markers. The assessment by the pathologist can already reveal crucial information regarding the differential CLAD diagnosis and prognosis. Because OB itself is challenging to detect on transbronchial biopsy (found in 2% of transbronchial biopsies, whereas 39% were considered ungradable[92]), other surrogate (risk) factors have been identified. Paraskeva et al[25] were the first to define acute fibrinoid organizing pneumonia, defined as the presence of intra-alveolar fibrin in the form of fibrin "balls" within the alveolar spaces, based on histopathological assessment, which was associated with a rapid decline in pulmonary function and death. This was later on confirmed in a small[93] and larger case series wherein specifically late-onset (90 d posttransplant) acute fibrinoid organizing pneumonia was found in approximately 10% of patients and was associated with a high risk of CLAD development, specifically RAS.[26] Late-onset organizing pneumonia and diffuse alveolar damage were also strongly related to CLAD.[27] Eosinophils on transbronchial biopsies also recently emerged as an interesting biopsy marker. Almost 8% of patients demonstrated a marked presence of eosinophils on transbronchial biopsies, which was associated with an increased risk for CLAD and death. No association was found with a particular CLAD phenotype.[28] Next to this expert pathology assessment, molecular methods are also gradually finding their way into routine clinical care. Shorter telomere length, a marker of physiological aging, assessed via quantitative fluorescence in situ hybridization on airway epithelial cells in endobronchial biopsies was found to be associated with CLAD.[29] Whole-genome messenger RNA profiling was performed with microarrays in a prospective collection of almost 500 transbronchial biopsies, in which a CLAD signature manifested not as inflammation but as parenchymal response-to-wounding associated with development, angiogenesis, and epithelial response-to-wounding in pathway analysis. Additionally, fibrillar collagen genes were increased in CLAD, indicating matrix changes, whereas normal transcripts were decreased.[30] Additionally, a molecular signature related to T-cell–mediated rejection detected either in transbronchial or mucosal biopsies was independently associated with graft loss (death or redo transplantation).[31]

Isolation and specific analysis of the different histologic patterns also bears great potential to discover novel and important biomarkers of CLAD. Specific excision and analysis of OB lesions revealed an important profibrotic signature with upregulation of different collagens and matrix remodeling pathways.[32] A combination of dysregulated proteins involved in the transforming growth factor-β pathway (bone morphogenetic protein 4, interleukin [IL]-6, matrix metalloproteinase 1, mothers against decapentaplegic family-1, and thrombospondin-1) has been proposed as predictor of CLAD.[33] An alveolar fibroelastosis pattern was also found to present with a molecular pattern of epithelial cell and fibroblast migration, macrophage differentiation, activation of epithelial-mesenchymal transition, neo-angiogenesis, migration of endothelial cells, and fibrogenesis compared with control lungs.[34] Interestingly, a comparison of the bronchial inflammatory gene expression score of airway brushes and transbronchial biopsies showed that the airway brushes outperformed transbronchial biopsies and improved detection of graft failure.[94] Expression of the proinflammatory mitogen- and stress-activated kinase 1 messenger RNA was found to be 2.9-fold higher in lung biopsies of patients 6 mo before CLAD diagnosis compared with stable patients.[95]

MARKERS IN BRONCHOALVEOLAR LAVAGE

A lung transplant patient is regularly subjected to a bronchoscopy with BAL to inspect sutures and to investigate potential signs of rejection or infection. A recent International Society for Heart and Lung Transplantation consensus guidelines attempted to harmonize the sampling of BAL and suggested an optimal protocol, which would also allow multicenter studies investigating biomarkers in BAL.[96] Usually, the BAL fluid is spun down, where the cell pellet can be used to investigate cellular content, molecular signatures, or microbial presence, whereas the supernatans can be used for measuring differential protein expression. The increased presence of mesenchymal colony-forming units (≥10 per 2×10^6 cultured mononuclear cells) in BAL assessed by culturing was associated with shorter CLAD-free survival.[35] Additionally, this increase in mesenchymal colony forming units was more frequently found in RAS patients, leading to a worse survival, independent of the CLAD phenotype.[36]

Another cell type that recently sparked interest is the eosinophil. Similarly, as observed on transbronchial biopsies, an elevated number of eosinophils in BAL (≥2%) is associated with a shorter CLAD-free survival, the RAS phenotype of CLAD and a shorter graft survival.[38] In patients already diagnosed with a RAS phenotype of CLAD, a higher percentage of eosinophils in BAL (cutoff ≥2%)

portended a worse outcome compared with patients with RAS without elevated eosinophils.[37] Natural killer (NK) cells could also be an important biomarker as these cells may promote tolerance by depletion of donor antigen-presenting cells. In patients with CLAD, NK cell in BAL were decreased, but these NK cells had a more activated phenotype.[39] Additionally, an increase in a specific subtype of NK cells expressing the natural killer cell group 2 isoform C receptor in BAL, indicative of response to cytomegalovirus infection, is associated with an increased risk of CLAD or death.[97] Autotaxin-expressing alveolar macrophages can also be detected in BAL of CLAD patients, which potentially drive mesenchymal stromal cell recruitment and tissue contraction in CLAD.[98]

Similar to the previously mentioned molecular signatures in biopsies, BAL signatures predictive of CLAD have also been investigated. Weigt et al[40] compared the transcription profile at 1-y posttransplant in patients with incipient CLAD and CLAD-free patients and found a profile skewed toward immune response pathways, dominated by genes related to recruitment, retention, activation, and proliferation of cytotoxic lymphocytes (CD8+ T cells and NK cells) in incipient CLAD cases. Additionally, an airway transcriptome signature derived from bronchial brushings and BAL revealed a type 1 immune response with predominant interferon-gamma, tumor necrosis factor alpha, and IL-1β expression in patients with CLAD.[41]

Foremost, BAL has been used to measure the expression of proteins, which play a role in the pathophysiological mechanism of CLAD either in the immunologic response or the response to injury and have been proposed as biomarkers. Comparing the expression of individual proteins in RAS compared with BOS and control demonstrated that only IL-6 was persistently significantly elevated in RAS compared with control and BOS patients.[42] Another study not only confirmed that elevated IL-6 and soluble IL-6 receptor were detected in BAL of CLAD patients but also revealed that these levels were raised in pre-CLAD samples, suggesting a role for the IL-6/IL-6 receptor pathway, which is in line with studies in kidney, heart, and liver transplantation.[43] M65 released during epithelial cell apoptosis and total cell death was found to be higher in RAS compared with BOS and control and associated with a worse CLAD-free survival.[44] To elucidate the pathophysiological pathway from an immunologic perspective, it is likely that a single marker will likely not suffice and that panels of markers could significantly aid in increasing the sensitivity and specificity for CLAD diagnosis. For example, alveolar alarmins (S100A9, S100A8/A9, S100A12, S100P, and high mobility group box 1), involved in apoptosis and inflammatory pathways were higher in BAL of RAS patients compared with BOS and control.[45] Prolonged elevation of C-X-C Motif Chemokine Receptor 3 chemokines (C-X-C Motif Chemokine Ligand 9, C-X-C Motif Chemokine Ligand 10, C-X-C Motif Chemokine Ligand 11) chemokines in serial BAL fluid measurements predicted the development of CLAD.[46] The combination of measures of C-X-C Motif Chemokine Receptor 3 chemokines in BAL and histopathologic patterns of acute rejection and organizing pneumonia proved to be more robust in predicting CLAD development, further emphasizing the importance of measuring these biomarkers in BAL, especially in combination with concurrent biopsy data.[47,48] Although single peptides from the renin-angiotensin system only tended to associate with CLAD, a combination of 7 proteins involved in the renin-angiotensin system separated patients with CLAD from stable patients.[49] Analysis of BAL samples also led to the discovery of an augmented protein expression level of humoral immunity in RAS (total IgG, IgG1–IgG4, and IgM) compared with BOS and control.[50] Next to markers related to inflammation, markers of aspiration can also be measured. Elevated levels of bile acids and predominance of conjugated species identified in bronchial washings were also independently associated with CLAD and graft survival.[51]

Lastly, BAL can be used to investigate the presence of distinct microbiota within the airways. Colonization and infection with *Pseudomonas aeruginosa* or *Aspergillus fumigatus* has been known to be associated with a shorter CLAD-free and overall survival for a long time.[99] The advent of new sequencing tools that allow the detection of microbial abundance and distribution in much more detail have clearly outlined the importance of distinct microbial signatures, which can serve as a biomarker for CLAD. A combination of culture-dependent and culture-independent methods showed that the posttransplant microbiota composition is highly variable in their bacterial load and community composition with many transient and low abundant taxa. However, a few microbes are present with a relatively high prevalence and/or abundance indicative that these could be important colonizers.[52] Additionally, the authors showed the existence of different microbial states with a balanced mode, characterized by a diverse bacterial community and relatively low viral load, and 3 other modes in which there is either bacterial depletion or dominance of potential pathogens. The balanced mode is likely related to immune tolerance, whereas the others states are associated with increased immune activity, lower respiratory function, and increased risks of infection and rejection,[52] which was also conclusively demonstrated in an experimental setting.[53] A comprehensive study furthermore demonstrated that the microbial signature outperformed BAL cellular composition, metabolome, or lipidome as predictor of future CLAD wherein a specific strong association was found between FEV_1 changes and *Capnocytophaga gingivalis* and *Veillonella dispar*.[54] Another study found that CLAD is associated with increased bacterial biomass and a Proteobacteria-enriched airway microbiome and epithelial to mesenchymal transition via N-myc-interactor expression.[55] Other studies, however, failed in pinpointing a certain pathogen to be responsible for the increased risk of CLAD. Indeed, Combs et al[56] showed that the microbial composition 1-y posttransplant was different in patients who will die or develop CLAD in the next 500 days compared with CLAD-free and surviving patients but could not relate this to a difference in individual bacterial taxa. Another study failed to find an association between the microbial composition and CLAD or CLAD phenotypes.[100]

Next to the bacterial composition, there is also an important association between viral infection and CLAD with a specific strong association with respiratory syncytial virus, parainfluenza virus, and human metapneumovirus.[101,102] However, also next-generation techniques are increasingly available, which can detect viral presence and the virome.

Metagenomic next-generation sequencing can also identify RNA viruses within the human pulmonary virome, including novel RNA viruses,[103] although their importance with regards to CLAD remains to be established.

CIRCULATING MARKERS

Although BAL and tissue markers provide an unclouded view on the ongoing processes in the lung microenvironment, the invasiveness of the procedure and the differences in clinical practices hamper their consistent use. For these reasons, circulating markers are an important area of ongoing research where promising biomarkers have already been discovered. Far along the track from discovery to universal use as a biomarker are donor-specific antibodies (DSAs). The proportion of patients in whom DSAs are found is variable across different centers, and although DSAs were historically typically associated with antibody-mediated rejection, it is clear nowadays that DSAs are universally associated with a higher risk for future CLAD development and mortality. In recent years, this has been further refined. For example, although persistence of DSA associate with all CLAD forms, the association with the RAS phenotype of CLAD is much stronger.[57,58] Evidence also arose that specifically HLA-DQ antibodies are a prognostic important marker associated with a worse CLAD-free survival,[59,60] whereas complement-binding DSA (typically C1Q+) is also associated with shorter CLAD-free survival.[60,61] Next to antibodies that are specifically directed to the donor, antibodies to self-antigens (ie, collagen V, K-α 1 tubulin, angiotensin type 1 receptor, and endothelin type A receptor) can also be found in the circulation, which elicit an auto immune response leading to a higher incidence of CLAD.[62] Recently, it also became clear that circulating exosomes, nanovesicles shown to regulate physiological processes in vivo, isolated from plasma by ultracentrifugation, can also be an important biomarker for CLAD. The exosomes derived from plasma from BOS, and RAS patients showed distinct molecular and immunologic profiles with exosomes from RAS patients typically demonstrating a higher concentration of proinflammatory factors, lung self-antigens, and antibodies to HLA class II molecules.[104] Interestingly, serial analysis in a cohort of pediatric and adult lung transplant patients demonstrated that exosomes containing self-antigens are detectable in the circulation before BOS onset.[63,64] Donor-derived cell-free DNA (ddCfDNA) is another promising marker that is getting closer to routine clinical care, possibly in combination with other makers.[105] These are short fragments from nuclear and mitochondrial DNA that are produced during apoptosis, necrosis, or by active secretion from cells. Using shotgun sequencing on plasma samples, a percentage of ddCfDNA being present in the sample can be calculated, and patients having levels in the highest tertile showed a significant higher chance of developing allograft failure (a combined end-point of CLAD, redo transplantation or mortality).[65] However, the question that remains to be resolved is whether this is due to the strong association with established risk factors for CLAD. Indeed, ddcfDNA is 6-fold higher in patients experiencing an acute rejection, showing a strong association with the histologic grade of rejection and the pulmonary function decline.[66] Similarly, the %ddcfDNA is higher among patients in whom a microbe was present with concurrent abnormal histopathology and those patients in which a higher risk pathogen (ie, *P. aeruginosa*, *A. fumigatus*, parainfluenza virus among others) is found.[67] Also, in patients with primary graft dysfunction, higher levels of ddcfDNA were found than in non-PGD patients, and these higher levels were subsequently related to a higher risk of developing CLAD.[68] Other peripheral markers are further away from routine clinical care but nevertheless could be interesting to further investigate and develop, especially because these will also provide insight into the underlying pathophysiological mechanisms of CLAD. The profile of acute phase proteins, for example, may also help to predict future CLAD development as, for example, alpha-2-macroglobulin was found to be an independent predictor of CLAD.[69]

Cell types that can be found in the peripheral blood either by a simple cell differential count or by cell sorting can have a protective or deleterious role in CLAD development. Neutrophils in peripheral blood, for example, demonstrated a high increase postoperatively and a subsequent progressive decrease until normal range. However, those recipients with CLAD had higher neutrophil counts and a slower return to normal levels.[70] Also, eosinophil cell counts above the normal range (defined as 8%) are associated with CLAD, especially RAS and worse posttransplant outcome, similar as the previously described association between the eosinophil counts in BAL or presence of eosinophilia on transbronchial biopsy and subsequent CLAD development. Interestingly, the peak blood eosinophil count was found to be predictive of CLAD-free and graft survival, independent of BAL eosinophil counts, making this a possibly relevant cell type that requires further in-depth investigation.[71] Additionally, BOS is associated with increased peripheral blood NK and natural killer T-cell-like cells expressing granzymes, perforin, and T-helper 1 proinflammatory cytokines.[72,73]

IL-10-secreting CD24hi CD38hi transitional B cells expressing CD9 are also associated with a better allograft outcome in lung transplant recipients.[74] Another interesting, but yet mostly elusive, cell type are the regulatory T cells. An increased proportion of circulating $CD4^{+}CD25hiFoxP3+$ T cells early posttransplantation is found in lung transplant patients who proceed to develop BOS within 3 y.[75] Similarly, increased frequency of CD4+ CD25 high CD127 low T cells early after lung transplant is associated with improved CLAD-free survival.[76] T-reg cell counts progressively decreased according to the severity of CLAD, further indicating their potential role as a biomarker for CLAD.[77] Increased proportion of IL-2 producing T-cell type ($CD4^{+}CD57+ILT2+$ T cells) over the first year posttransplant also predicted CLAD.[78] Also, regulatory B cells defined as CD19+CD24highCD38high B cells are believed to participate in long-term lung graft acceptance,[79] although more evidence is needed to evaluate their potential role as a biomarker in transplant tolerance.

The role of telomeres was already previously discussed in lung biopsies, wherein shorter telomere lengths on biopsy were associated with shorter CLAD-free survival, however, telomere lengths can be assessed more easily in circulating blood. Leukocyte telomere length shortening (defined as shorter than 10th percentile) was found in approximately 32% of recipients with pulmonary fibrosis before transplantation and was associated with a shorter CLAD-free and overall survival[80] possibly via an impaired CD8+ T-cell proliferation to alloantigens.[81] These findings were later also confirmed by another study demonstrating an association

between telomere shortening and clinical significant leukopenia and reduced CLAD-free survival.[82] Krebs von den Lungen-6 (KL-6), a glycoprotein expressed on pulmonary epithelial cells, is a marker that is being investigated in the field of interstitial lung diseases and that has already been proposed as a potential biomarker for lung rejection in 2006 as serum levels were higher in lung transplant patients with BOS versus patients without BOS and healthy controls.[83] Moreover, serum KL-6 levels also correlated with the FEV_1 decline.[84] Serial analysis of KL-6 levels demonstrated that an increase in KL-6 was associated with CLAD.[85] More recently, Berastegui et al[86] demonstrated that KL-6 levels were specifically higher in patients with RAS compared with BOS patients, wherein these levels also correlated with the decline in FVC in the 6 mo following sampling.

PRIME TIME?

Adequate phenotyping of CLAD in patients is crucial in determining prognosis and treatment of CLAD patients. However, it also clear that adequate phenotyping based on physiologic and radiologic criteria remains troublesome despite recent international consensus guidelines as a subset of patients are still unclassified, and it is known that patients can switch CLAD phenotype at any given time during their follow-up. Moreover, the question remains as to when fibrosis on radiology is sufficient to be the culprit of the pulmonary function decline as a significant proportion of clinically defined BOS patients show evidence of fibrosis upon in-depth histopathologic assessment of lung explants.[106] Additionally, predicting CLAD before it clinically manifest could be important for earlier and more aggressive treatment of underlying risk factors.

This is where adequate biomarkers could really play an important role by predicting CLAD development by providing an accurate differential diagnosis and prognosis estimation. In that aspect, there remains a lot of ground to cover. A biomarker should bear a high sensitivity and specificity, should be ideally noninvasive and cost-effective, and despite huge efforts, there is none that stands out at the moment. Circulating DSAs are the most far along the road from discovery to universal usage given their strong association with poor outcome and the fact that these can be measured in blood. However, measurement of graft residing DSA in concomitantly taken transbronchial biopsies provide a lot of complementary information and identifies specifically those pathogenic DSA constituting an inferior outcome.[107] Another study in end-stage CLAD explant lungs demonstrated that graft DSAs were also present in patients without circulating DSAs, indicating that the lung could also serve as a sponge, absorbing these antibodies without significant DSA in the blood.[108] This can be seen as an example of the issues that are currently still faced in all proposed biomarkers and why biomarkers are not (yet) ready for prime time: there is not a single one that can be used by itself and that can provide information that can be used in clinical decision-making. The future will likely be in the use of a panel of markers in conjunction with clinically relevant information. Multicenter validation will be crucial in that aspect. Nowadays, most studies remain limited by their monocentric design and a narrow focus on one or several key molecules or markers. The recent consensus documents on CLAD and RAS provide a solid framework to clinically characterize the patients, and initiatives to unify the approaches on sample taking and processing as exemplified by the recent BAL consensus are key in initiating multicenter studies that could provide sufficient power and a strong scientific basis to translate key findings back to the routine clinical care of the patient.

Ultimately, it is also key to select the appropriate endpoints that a biomarker should predict, and given the diversity in biological pathways and the difference in clinical presentation and outcome, using CLAD as an end-point in studies is probably not sufficient anymore. Biomarker studies should instead rather focus on rigorous dissection of BOS and RAS patients and investigate the relative contribution to the prediction or diagnosis of these specific phenotypes. In that aspect, it is also important to realize that once CLAD is diagnosed, the window of opportunity for therapeutic intervention is much shorter, and research initiatives should focus on earlier identification of patients at increased risk, which allows the earlier initiation of therapeutic interventions, which is where an appropriate biomarker would really be of tremendous value.

In conclusion, although significant efforts have been made, most biomarkers have not made it to clinical routine. Current research is increasingly focusing on the combination of different biomarkers, which is probably where the future lies. Ultimately, the search for a biomarker panel is still on and could significantly assist in the management of patients with CLAD.

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