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Review article

# The potential of biomarkers of fibrosis in chronic lung allograft dysfunction

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## ARTICLE INFO

## ABSTRACT

Chronic lung allograft dysfunction (CLAD) is the major long-term cause of morbidity and mortality after lung transplantation. Both bronchiolitis obliterans syndrome and restrictive lung allograft syndrome, two main types of CLAD, lead to fibrosis in either the small airways or alveoli and pleura. Pathological pathways in CLAD and other types of fibrosis, for example idiopathic pulmonary fibrosis, are assumed to overlap and therefore fibrosis biomarkers could aid in the early detection of CLAD. These biomarkers could help to differentiate between different phenotypes of CLAD and could, in comparison to biomarkers of inflammation, possibly distinguish an infectious event from CLAD when a decline in lung function is present. This review gives an overview of known CLAD specific biomarkers, describes new promising fibrosis biomarkers currently investigated in other types of fibrosis, and discusses the possible use of these fibrosis biomarkers for CLAD.

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## Contents

## 1. Introduction

Chronic lung allograft dysfunction (CLAD) is the main complication limiting long term survival in lung transplantation [1]. It is estimated that about 50% of lung transplant recipients develop CLAD in the first five years after transplantation [2] and that development of the disease is associated with lower quality of life [3]. CLAD remains the primary cause of death in half of all lung transplantation(LTx) patients [4] even though surgical procedures and immunosuppression have improved over the last years. CLAD includes two distinct forms of graft dysfunction, restrictive allograft syndrome (RAS) and bronchiolitis obliterans syndrome (BOS), the latter being the most common [5]. BOS is characterized by remodeling and fibrotic obliteration of small airways and it is thought to have a multifactorial origin. Repeated airway injury induces recruitment of inflammatory cells that stimulate transition of epithelial cells into mesenchymal cells, migration of myofibroblasts and production of extracellular matrix leading to occlusion of segments of small airways [6]. Since histological confirmation of BOS is difficult because of its variable appearance, BOS is a clinical diagnosis based on lung function decline [7,8]. Currently, BOS is diagnosed through spirometry with measurement of forced expired volume in one second (FEV1) and FEV1/forced vital capacity (FVC). BOS is confirmed when a 20% decline in FEV1 compared to baseline persists for at least three months, after exclusion of other causes of FEV1 decline, for example infection or acute rejection [9]. Early detection of BOS is essential, since further decline and mortality can possibly be prevented with changes in treatment regimen [7,10].

In 2011 RAS, a more progressive form of CLAD was identified by Sato et al. Patients with a RAS phenotype exhibit peripheral lung fibrosis and restrictive lung function [11], negatively influencing survival compared to BOS [12]. Diagnosis of RAS is based on a persistent 20% decline in FEV1 for at least three months with a concomitant decline of at least 10% in total lung capacity. Moreover, persistent parenchymal opacities with or without pleural based opacities of the chest should be visible on high resolution computed tomography or chest x-ray [13]. The overall survival for RAS patients is estimated around 6–18 months, compared to 3–5 years for BOS, necessitating a correct diagnosis for patient consultation as well as for research purposes [14–16].

Biomarkers could aid in unravelling the underlying pathophysiology of CLAD. Importantly, biomarkers may have a place in differentiating between BOS and RAS and play a role in identifying likelihood of rapid disease progression. Different specimens have been used to detect biomarkers in CLAD: serum, bronchoalveolar lavage fluid (BALf) and trans bronchial biopsies being the most common. Preferably, the specimen is collected via a simple technique, for example blood sampling.

In the early phase of disease, fibrosis is observed in patients with CLAD together with inflammation. Therefore, many studies focus on inflammation and genetic polymorphisms as possible biomarkers for CLAD. Inflammation, however, is highly dependent on other factors like infection or gastro-esophageal reflux. Since fibrosis is an active process, independent, but interconnected with inflammation, that progresses during advancing stages of CLAD, fibrotic biomarkers may serve as robust markers for early detection and disease progression. In addition, many pathological pathways in other fibrotic diseases, like idiopathic pulmonary fibrosis (IPF), overlap with CLAD development. It may therefore be of great help to evaluate biomarkers that have been studied for these conditions [17,18]. Several biomarkers appear to be suitable to detect fibrosis in multiple fibrotic diseases in different organ systems like IPF, auto-immune disorders and liver fibrosis

[19–22]. In this review we give an overview of known CLAD specific biomarkers and describe new promising fibrosis biomarkers, currently studied in other types of fibrosis, that may also be useful in CLAD.

## 2. Pathophysiology of CLAD

After LTx, allo-immune-dependent and independent factors are believed to start a response that ultimately culminates in airway or interstitial fibrosis (Fig. 1). Allo-immune dependent factors include immune responses of CD4+ T cells that are highly sensitive to mismatched major histocompatibility complex (MHC) class I and class II. Also, immune responses are initiated by antibodies against MHCI or several non-MHC antigens, such as an antibody against alpha1-tubulin which targets epithelial cells. Independent factors that are believed to contribute to occurrence of BOS are infections, gastro-esophageal reflux and primary graft dysfunction [23–25].

Central in the process leading to airway or interstitial fibrosis is continuous epithelial or endothelial micro-injury, by allo-reactive T cells, antibody-activated natural killer cells or macrophages, infections, acid, among others, resulting in activation of innate immune responses and complement. Cytokines and chemokines released by resident immune and lung cells will attract and activate neutrophils, monocytes and macrophages in an attempt to mitigate injury and start tissue repair (Fig. 1). Important in the repair process are interactions between macrophages and (myo)fibroblasts that spiral into excessive production of extracellular matrix in response to continuous micro-injuries leading to the fibrotic abnormalities seen in CLAD. Macrophages produce several pro-fibrotic growth factors like transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF) and interleukin (IL)13, that activate lung-resident fibroblasts and induce their differentiation into myofibroblasts. These are the main producers of the excess extracellular matrix that is central to all types of CLAD [26]. The myofibroblast population does not solely originate from resident lung fibroblasts. Fibrocytes, monocytic precursors of fibroblasts found in blood, are attracted to peripheral tissue by several chemokines and can differentiate into fibroblasts [27]. Furthermore, epithelial cells can differentiate into myofibroblasts through a process called epithelial-mesenchymal transition possibly through activation by TGF-beta [28].

Whereas BOS is characterized by fibrotic changes to the airways and their subsequent occlusion, RAS encompasses anomalous obliteration of alveoli by interstitial fibrosis perpetuated by myofibroblasts [6]. Recent studies show that in patients with RAS, TGF-beta also causes mesenchymal cells of the pleura to differentiate into myofibroblasts, through a process called mesenchymal-to-myofibroblast transition [18]. Incidentally, this process has also been identified in IPF patients [29,30].

The continuous micro-injury and repair/remodeling of the transplanted organ will of course leave its mark in several compartments of the body. Several markers of these processes have already been studied in CLAD in serum as well as in BALf. For example, exosomes containing high concentrations of self-antigens to k-α 1 tubulin (K-α1T) and collagen type V (colV) were found in serum and BALf of patients with BOS. These exosomes represent injury but possibly also act as immunologically active particles that activate the recipients immune system [31,32].

Different fibrotic diseases may share common underlying mechanisms reflecting a wounding response to injury. This response characterizes the complex nature of fibrosis with involvement of effector cells such as fibroblasts, myofibroblasts and fibrocytes and deposition of extracellular matrix [33]. Hypothetically biomarkers studied in IPF
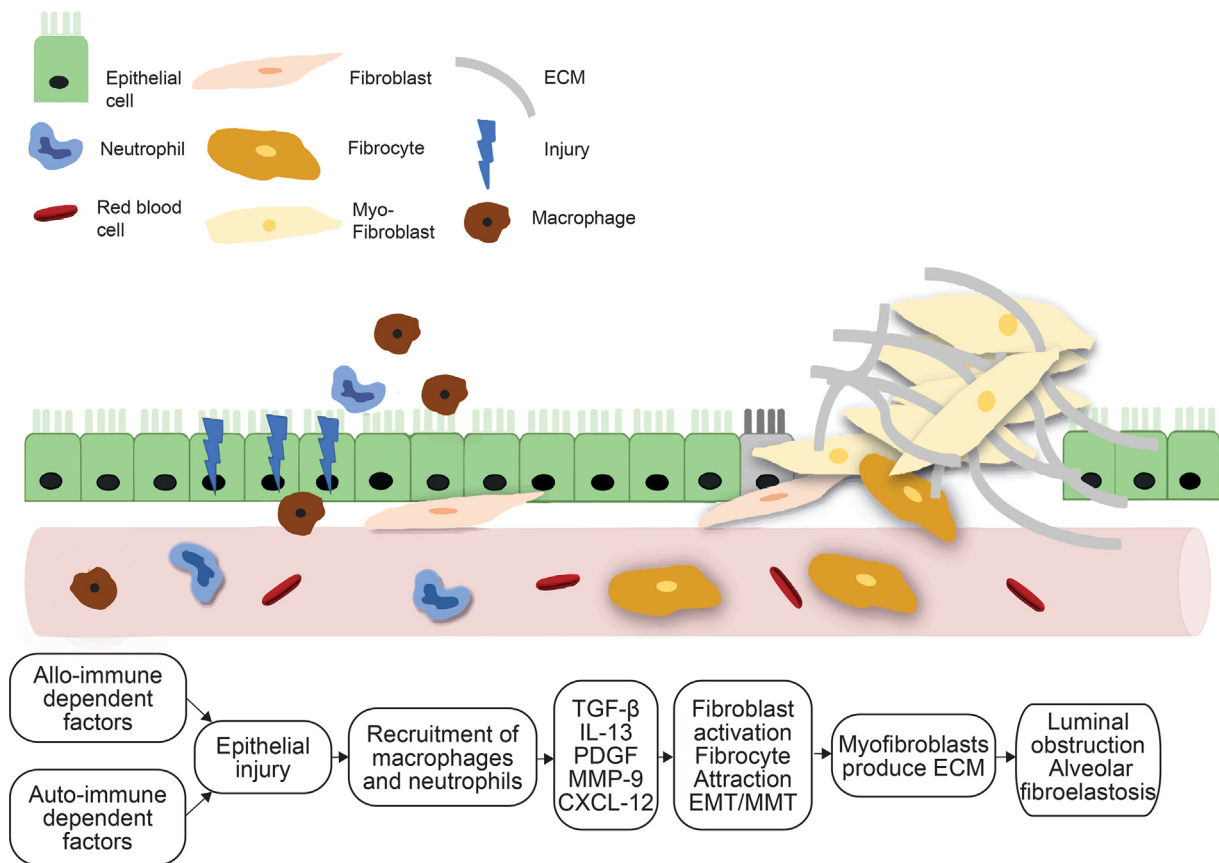
**Fig. 1.** Pathway of formation of extracellular matrix causing luminal obstruction in BOS (bronchiolitis obliterans syndrome) or interstitial fibrosis in RAS (restrictive allograft syndrome). Abbreviations: IL-13, Interleukin 13; TGF-β, transforming growth factor beta; PDGF, platelet derived growth factor; MMP-9, matrix metalloproteinase 9; CXCL12, stromal cell derived factor 1; EMT, epithelial mesenchymal transition; MMT, macrophage to myofibroblast transition; ECM, extracellular matrix.

and other fibrotic diseases like liver cirrhosis could also act as biomarkers for CLAD. For example, markers known for their function in cell proliferation or extracellular matrix production like fibulin-1 [34], osteoprotegerin (OPG) [35] and pro-C3 [20] could also reflect manifestation of fibrosis in CLAD (Fig. 2). Since the anatomy of affected organs, the effector cells involved and ECM between tissues differ, the usefulness and comparability of fibrosis biomarkers in CLAD is unknown, needing further research.

## 3. Current biomarkers associated with fibrosis in CLAD

### 3.1. MMP-9

Matrix metalloproteinases (MMPs) constitute a large group of proteinases that are part of the process of turnover and stabilization of the extracellular matrix through their ability to degrade structural proteins like collagen. MMPs are inhibited by tissue inhibitors of MMPs (TIMPs) that bind the catalytic site of MMPs, leading to inactive MMPs. MMPs are produced in an inactive form by many different cell types and once activated in the extracellular space are thought to carry out different functions depending on the cell type that produces them [36]. Since MMPs not only cleave structural proteins in the extracellular matrix but also cleave signaling proteins like cytokines, they can actively influence the inflammatory state of tissue and indirectly influence the pro-fibrotic activity of resident and immune cells(Fig. 2) [37]. MMPs clearly have a function in extracellular matrix remodeling, but are also studied as biomarkers for IPF, cardiovascular disease and cancer [38–40].

MMP-9, a gelatin degrading MMP, has a clear link with fibrotic remodeling. It is mainly produced by innate immune cells like neutrophils, eosinophils, and monocytes/macrophages but also by epithelial

cells [41]. Fernandez et al. used MMP-9 knockout mice in a tracheal allograft transplant model and observed that obliterative allograft dysfunction was reduced in these mice compared to wildtype mice. MMP-9 also modulated cellular infiltration into the allograft and activation and expansion of alloreactive T-cells. Furthermore, treatment with doxycycline, a known inhibitor of MMPs, delayed development of BOS in mice [42]. Not only MMP-9 appears to be involved in fibrotic changes, other MMPs may also have a role.

Sato et al. showed that inhibition of MMPs with a low dose broad spectrum MMP inhibitor (SC080) increased degradation of an established collagen matrix in a rat intrapulmonary tracheal transplant model, an effect that was not observed with a high dose of the inhibitor. This seems counter intuitive, however when transgenic deletion or low dose chemical inhibition of MMPs was applied, upregulation of collagenolytic MMPs, like MMP-2, was induced. This consequently increased degradation of the established collagen matrix and lowered production of collagen by myofibroblasts. Strikingly, expression of MMP-8, −9 and − 13 was reduced, further illustrating the complexity of the response in CLAD, in concert with observations that MMPs can be up- and down- regulated in IPF, with their function being dependent on their tissue location [43]. Reduced T-cell infiltration into the fibrous deposit in the occluded airway through low dose SC080 treatment coupled with lower numbers of myofibroblasts, augmented the effect. When low dose SC080 treatment was combined with cyclosporin treatment, a reduction in obliteration of allograft airways was observed [44]. In LTX patients, sputum MMP-9 and TIMP-1 levels were significantly higher compared to normal controls, with CLAD patients having the highest levels of MMP-9. In addition, the MMP-9/TIMP-1 ratio was high in patients with chronic rejection, suggesting an imbalance between MMP-9 and its inhibitor that may lead to aberrant extracellular
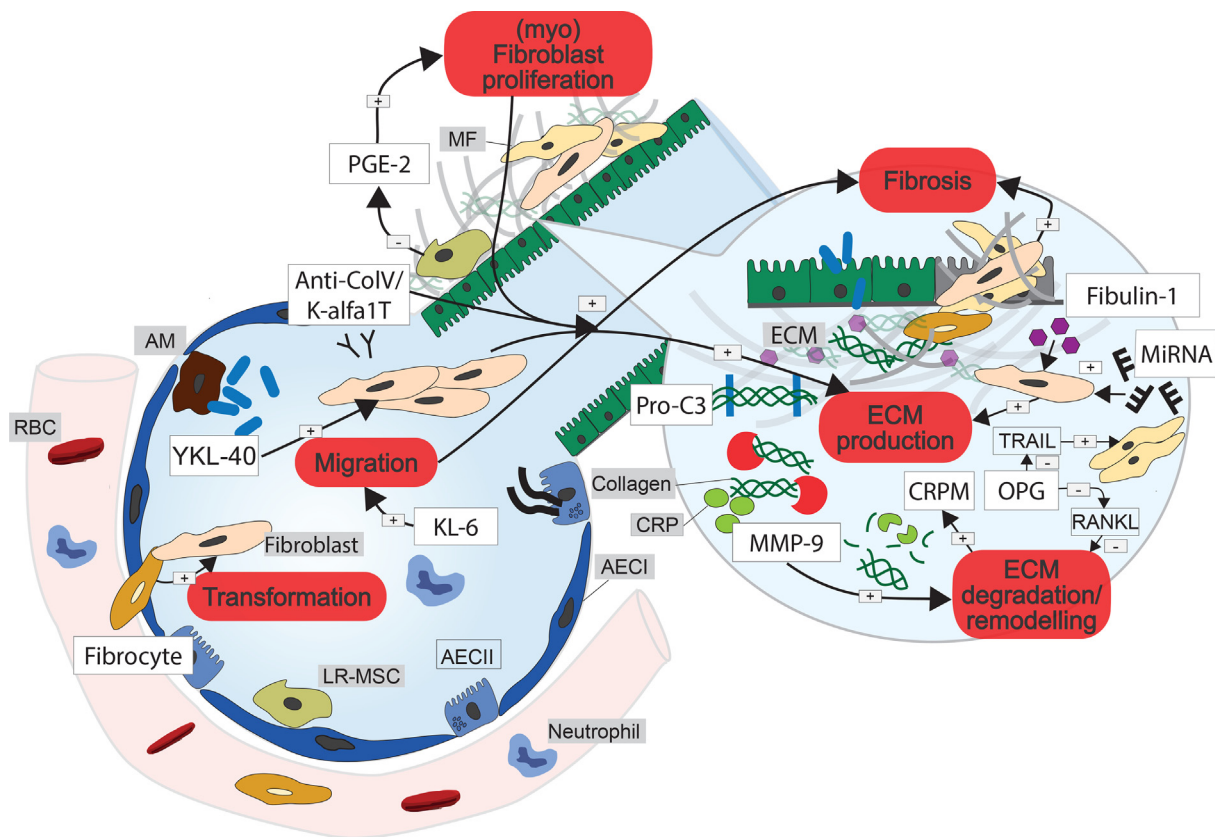
**Fig. 2.** Current and possible biomarkers and accompanying processes leading to fibrosis in chronic lung allograft dysfunction. Abbreviations: ECM, extracellular matrix; CRC-reactive protein; CRP-M, C-reactive protein degraded by MMP; OPG, osteoprotegerin; MiRNA, microRNA; anti-ColV/K- $\alpha$1T, antibodies to collagen V and K- $\alpha$ 1 tubulin; AM, alveolar macrophage; AEI, alveolar epithelial cell type I; AEII, alveolar epithelial cell type II; LR-MSC, lung resident mesenchymal stromal cell; RBC, red blood cell.

matrix remodeling in the airways, activation of growth factors and cytokines and the induction of a profibrotic response as is characteristic of this MMP [43,45,46]. Importantly, a study by Hubner et al. reported a higher MMP-9/TIMP-1 ratio in patients with BOS and this ratio also correlated negatively with FEV1 in these patients [47].

In acute asthma, sputum MMP-9 is high during onset of an exacerbation with low TIMP-1 levels and thus high MMP-9/TIMP-1 ratios, suggesting an inflammatory role for MMP-9 [48].

Recently, Pain et al. showed that plasma levels of MMP-9 also predicted onset of BOS and RAS a year before diagnosis. Levels in plasma increased before diagnosis, but declined with disease progression suggesting that the involvement of MMP-9 is primarily in the early phases of CLAD [49]. In concordance, Kasteleijn et al. observed high MMP-9 serum levels in BOS positive patients shortly after transplantation, however no longitudinal change was seen in the MMP-9 levels [50].

MMP-9 levels in sputum rise before onset of CLAD, which is why MMP-9 could serve as a biomarker for CLAD. However, the influence of TIMP-1 levels and its relation to MMP-9 is not yet clear and study populations thus far have been small thereby limiting interpretation of the significance in CLAD.

### 3.2. YKL 40

YKL-40 is a chitinase-like glycoprotein that is expressed and secreted by lung alveolar macrophages, neutrophils and epithelial cells. Its exact function is unknown, however YKL-40 is known to be a connective tissue growth factor that promotes fibroblast growth and contributes to tissue remodeling and degradation of extracellular matrix [51,52] (Fig. 2). In addition, YKL-40 binds to type 1 collagen

and stimulates its fibril formation [53]. Higher YKL-40 levels are recognized to reflect the fibrogenic process in fibrotic diseases like liver fibrosis, cryptogenic obliterative pneumonia and remodeling of airways in asthma [51,54,55]. Furuhashi et al. reported a higher expression of YKL-40 in serum and bronchial epithelial cells of patients with IPF. In particular, YKL-40 expression is recognized in the bronchiolar epithelial cells lining honeycomb spaces and in intra-alveolar spaces bordering fibrotic lesions, suggesting a contributing role in development of fibrosis and tissue remodeling [56]. Interestingly, YKL-40 levels in BALF and serum do not correlate, which could possibly be explained by different sources of YKL-40 (alveolar macrophages and epithelial cells for YKL-40 levels in BALf versus peripheral immune cells contributing to YKL-40 levels in serum) and thus possibly reflecting two distinct processes [56,57]. In LTx patients, YKL-40 serum levels before LTx are significantly higher in patients who develop BOS after LTx compared to those who do not and identify patients at risk for development of BOS [21] (Table 1).

YKL-40 levels remain significantly higher in patients developing BOS and are also higher during progression of disease. In contrast, another study in 20 LTx patients, reported no differences in serum levels of YKL-40 between BOS-positive and BOS-negative patients measured between date of transplantation and date of onset of BOS. These investigators also found no longitudinal changes in serum levels of YKL-40 preceding BOS [50]. These opposing results may be explained by promoter polymorphisms in the gene encoding for YKL-40 in different populations. 10–23% of the variation in YKL-40 levels in healthy individuals could be explained by polymorphisms [58,59]. Therefore, more studies are necessary to investigate the role of YKL-40 and its predictive value for BOS in LTx patients. It would be of interest to investigate if different polymorphisms can indeed explain variations in serum levels before and after transplantation and if YKL-40 levels change with progression

**Table 1**
Normal levels of current biomarkers in healthy controls.

| Current biomarkers | Patient (n) | Specimen | Level in healthy controls | Levels in CLAD | Levels in Non-CLAD | Study |
|---|---|---|---|---|---|---|
| MMP-9 | 45 | Sputum | 41 ± 30 ng/ml | ↑↑↑ | ↑↑ | Beeh [45] |
| YKL-40 | 57 | Serum | 46 (33–71) ng/ml | ↑↑↑ | ↑ | Jaksch [21] |
| KL-6 | 46 | Serum | 235 ± 142 ng/ml | ↑↑ | ↑ | Bessa [68] |
|  | 73 | Serum | 265 (199–345) u/ml | ↑↑↑ | ↑ | Berastegui [70] |
| Fibrocytes | 39 | Serum | UK | ↑↑ | ↑ | Lapar [27] |
| Mir-144 | 39 | Lung tissue | UK | ↑↑ | ↑ | Xu [83] |
| Mir-151 | 33 | Serum | UK | ↑↑ | ↑ | Budding [80] |

Abbreviations: n, number; UK, unknown. Levels of biomarkers in BOS and non-BOS are presented as increase (↑) or decrease (↓) in lung transplantation patients compared to healthy controls.

of disease. In addition, further research into polymorphisms of YKL-40 is needed to elucidate if specific morphisms are associated with development of BOS or RAS.

### 3.3. KL-6

KL-6 is a glycoprotein that is mainly present on the surface of type II alveolar epithelial cells, but is also expressed by other lung epithelial cells. It acts as a chemotactic factor for human fibroblasts(Fig. 2) [60]. In healthy individuals a small amount of KL-6 is found in serum due to leakage from alveoli into the circulation. Higher KL-6 levels in serum after lung injury are believed to be the result of increased permeability of the blood/alveolar barrier and the high expression by regenerating type II alveolar epithelial cells. KL-6 induces fibrotic changes through its proliferative and anti-apoptoptic actions on fibroblasts [61,62].

KL-6 is elevated in people with interstitial lung disease and proved to be a useful marker of disease activity in interstitial lung disease [63–65]. Significant increases in KL-6 levels did not occur in acute rejection in LTX patients, which suggested it may be a useful marker to differentiate between BOS and acute rejection [66]. In 2006, Walter et al. studied the levels of KL-6 in serum of LTx patients and concluded that the levels of KL-6 were significantly higher in LTX patients with BOS compared to LTX patients without BOS and healthy individuals. Also higher levels of KL-6 correlated negatively with FEV1 [67]. Furthermore, KL-6 levels increased in time in LTX patients who developed CLAD, but declined in stable LTX patients 7 years after transplantation [68]. Later studies from Oshimo and colleagues showed that patients with RAS had significantly higher levels of KL-6 compared to BOS patients, perhaps due to increased injury to type II alveolar epithelial cells in RAS (Table 1). Therefore KL-6 could possibly be used to distinguish between BOS or RAS [69,70].

However, the study populations to date have been small and validation in larger cohorts is needed with use of more follow up samples to analyze the utility of changes of KL-6 longitudinally.

### 3.4. Fibrocytes

Fibrocytes are mesenchymal cell progenitors of myeloid origin that can migrate to areas of injury and transform into fibroblast-like cells that produce extracellular matrix. Classic fibrocyte cell surface markers are the stromal marker collagen-1 and the immune cell marker CD45+ [27]. The role of fibrocytes in several fibrotic lung diseases is already acknowledged [71,72]. Fibrocytes are thought to be recruited from the circulation through chemokine receptors responding to secreted chemokines released after alveolar damage. These chemokines attract fibrocytes to the site of injury and stimulate their production of collagen type I [73]. Moreover, fibrocyte secreted TGF-beta1 is known to activate resident fibroblasts, possibly aggravating fibrotic responses to lung injury.

In a cohort of 39 LTx patients, LaPar et al. described that the number of circulating fibrocytes in serum of BOS patients was significantly higher compared to non-BOS patients and correlated positively with advanced stages of BOS. In addition, fibrocytes expressing CXCR4, a specific subset of fibrocytes, significantly increased with advancing BOS stage [27]. Research in IPF and murine models in bleomycin-induced lung injury showed that CXCR4+ fibrocytes, attracted by the chemokine CXCL12, played a significant role in pathogenesis of fibrosis [74,75]. Therefore, this subset of fibrocytes may play a role in the pathogenesis of BOS through the CXCR4/CXCL12 axis, but further research is warranted to investigate this.

It is not known what the role is of fibrocytes in the pathogenesis of RAS and their use as a biomarker is therefore uncertain. Moreover, the role of underlying disease for transplantation on the number on fibrocytes in unilateral transplanted patients also requires further consideration.

### 3.5. Micro-RNAs

Micro RNAs(miRNA) are short non-coding RNA molecules that regulate protein expression at the post-transcriptional level through binding to target messenger RNAs and by preventing translation of these messenger RNAs [76]. miRNAs regulate more than 30% of protein gene coding and are assigned an important role in the regulation of major cellular processes such as cell development and epithelial mesenchymal transition [77,78]. Different miRNAs were either up- or downregulated in patients that develop antibodies to MHC molecules, a known risk factor for BOS, and appear to impact TGF-beta and B-cell receptor signaling pathways [79].

Several miRNAs associated with fibrosis have been investigated in CLAD. Pro-fibrotic mir-21 and mir-155 and anti-fibrotic mir-29a were elevated in serum prior to the diagnosis of BOS compared to non-BOS transplanted patients. Moreover, mir-103, a miRNA previously not associated with fibrosis, was also elevated in serum before diagnosis of BOS, possibly due to active release of miRNAs in exosomes or upon cell injury or death caused by a persistent inflammatory environment in the lung [80]. In a rat tracheal transplant model of obliterative bronchiolitis [81] and in pediatric transplantation patients with BOS [82], the gene for mir-155, which alters a target gene involved in B- and T-cell differentiation and function, was upregulated in BOS compared to non-BOS suggesting a potential role for mir-155 in the induction of an unwanted immune response against the donor organ leading to graft dysfunction.

The expression of mir-144 was higher in lung tissue of patients with BOS compared to non-BOS, while mir-144 regulated fibrogenesis (Fig. 2) in fibroblasts through increased activity of the TGF-beta/SMAD pathway. The role of mir-144 was confirmed by transfecting fibroblasts with mir-144 and finding that these then expressed more alpha smooth muscle actin and fibronectin, a sign of transformation to myofibroblasts [83]. To date, no studies have reported on the role of miRNAs in RAS.

## 4. Possible future biomarkers

### 4.1. Osteoprotegerin

Osteoprotegerin (OPG) is best known as a decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL) and tumor

necrosis factor-related apoptosis-inducing ligand (TRAIL). These are all members of the tumor necrosis factor receptor family and mostly studied in the context of bone homeostasis and cancer [84,85]. RANKL is expressed on osteoblasts and its receptor RANK is expressed on osteoclast precursor cells. Binding of RANKL to RANK on osteoclasts results in osteoclast activation and subsequent bone matrix degradation. OPG inhibits osteoclast activation and bone matrix degradation by neutralizing RANKL, thereby controlling bone density [86]. OPG can also neutralize TRAIL and thereby inhibit apoptosis induction by TRAIL which is most studied in the context of cancer [87].

OPG is not solely produced by osteoblasts and cancer cells, but is also known to be produced by epithelial cells, smooth muscle cells and (myo)fibroblasts. OPG messenger RNA and protein was found in lung tissue, especially during fibrotic lung injury in IPF and mouse models of pulmonary fibrosis [35,88,89] and OPG may be a predictor of rapid progression of IPF [35]. Earlier studies showed OPG to be an inducer of fibrogenesis by promoting vascular fibrosis and high expression of OPG was described in liver and cardiac fibrosis and chronic kidney disease [90–94]. In liver fibrosis, adding OPG to a panel of serum markers increased the diagnostic accuracy of this panel [95] (Table 2). Moreover, recent studies show that OPG expression also responded to antifibrotic treatment in vitro, suggesting OPG could not only be a biomarker for fibrosis severity but also for treatment efficacy [96]. The precise role of OPG in (pulmonary) fibrosis remains to be elucidated, but may involve neutralizing possible antifibrotic effects of RANKL and/or TRAIL. As RANKL induces degradation of extracellular matrix in bone, it may have similar effects in other tissues and neutralization of RANKL may prevent this. Deficiency in TRAIL was found to abrogate lung injury and fibrosis in bleomycin-treated mice and lower levels of TRAIL were found in patients with IPF [97]. It is suggested that excessive myofibroblast activation and extracellular matrix production is prevented by TRAIL-induced apoptosis of myofibroblasts. Therefore, neutralization of TRAIL by OPG may diminish these favorable effects of TRAIL, leading to progression of fibrosis [98] (Fig. 2). Summarizing,

given the close association of OPG with many types of fibrosis it could also possibly serve as a biomarker in CLAD.

### 4.2. Fibulin-1

Fibulin-1 is a glycoprotein produced by fibroblasts and airway smooth muscle cells [99] that aids stabilization of the extracellular matrix by binding to elastic fibers and interacting with fibronectin [100,101]. Fibulin-1 has been reported to stimulate, as well as inhibit, wound repair in different tissues. Four isoforms of fibulin-1 have been identified in humans; fibulin-1A, B, C, and D. It is assumed that all isoforms have their own specific role. Fibulin-1A and B are thought to have a role in embryonal development, having been detected at low levels in human placenta, but are not usually detectable in adults [102]. Fibulin-1D has anti-oncogenic and anti-invasive properties, while fibulin-1C interacts with factors regulating cell differentiation [103]. A high ratio of fibulin-1C/fibulin-1D is associated with more aggressive tumor types in ovarian cancer [104]. Currently, only fibulin-1C is known to have a role in lung tissue remodeling. A study by Lau et al. showed that fibulin-1 levels are higher in serum and BALf from people with asthma compared to those without [99]. Exaggerated proliferation was reported in airway smooth muscle cells derived from asthmatic compared to non-asthmatic volunteers corresponding with previous studies [105,106]. This exaggerated proliferation was abrogated when fibulin-1C was downregulated through use of a specific fibulin-1C antisense oligomer in the asthmatic airway smooth muscle cells. Interestingly, migration of cells was not affected, indicating that fibulin-1C exerts its effect on remodeling mainly through regulation of proliferation. Furthermore, induction of fibulin-1C mRNA expression by TGF-beta was higher in airway smooth muscle cells from asthmatic compared to non-asthmatic volunteers while the levels of induction of fibulin-1D expression did not differ [99]. This corresponds with another study showing that in asthmatics fibulin-1D expression in bronchial biopsies was lower than in non-asthmatic volunteers [107]. Lau et al. also

**Table 2**
Current and suggested biomarkers in chronic lung allograft dysfunction (CLAD).

| Biomarker | BOS/RAS | Specimen | Source | Function/product | Current field | References |
|---|---|---|---|---|---|---|
| MMP-9 | BOS | Serum, BALf, sputum | Macrophages, neutrophils | Turnover of collagen, stabilization of ECM | CLAD | [42,44,45,47,49,50] |
| YKL-40 | BOS | Serum, BALf | Lung alveolar macrophages, neutrophils, epithelial cells | Promote fibroblast growth, contribute to tissue remodeling, degradation of ECM Stimulates collagen type I fibril formation | CLAD | [21,50] |
| KL-6 | BOS/RAS* | Serum | Type II alveolar cells, Epithelial cells | Chemotactic factor for fibroblasts | CLAD | [67–70] |
| Fibrocytes | BOS | Serum | Bone marrow | Production of collagen I and III Transformation into (myo)fibroblast Secretion of TGF-beta1 | CLAD | [27] |
| Microrna | BOS | Serum, lung biopsy | Nucleus | Regulation of protein expression | CLAD | [79,81–83] |
| **Possible biomarkers** | | | | | | |
| Osteoprotegerin | Possible BOS/RAS | Serum | Osteoblasts, epithelial cells, smooth muscle cells, (myo)fibroblasts | Associated with fibrosis possibly via RANK/RANKL or TRAIL | Liver fibrosis, IPF | [35,90,91,94] |
| Fibulin-1 | Possible BOS/RAS | Serum | Fibroblasts | Stabilization ECM Function in wound repair | Asthma, IPF | [34,103,108] |
| CPRM | Possible BOS/RAS | Serum | C-reactive protein | C-reactive protein degraded by MMP-1/8 | IPF | [117] |
| Pro-C3 | Possible BOS/RAS | Serum | Collagen type III | Pro-C3 is a substitute marker of collagen type III formation | IPF, Liver fibrosis | [19,110,114] |
| Anti-Kα1T Anti-ColV | Possible BOS | Serum, BALf | Alveolar epithelial cells Collagen type V | Self-antigens that induce an immune response | CLAD | [121,122] |
| Prostaglandin E2 | Possible BOS/RAS | Unknown | Mesenchymal stromal cells | Inhibition of fibroblast migration, collagen synthesis and differentiation of myofibroblasts | CLAD | [129,130] |

Abbreviations: BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; BALf, bronchoalveolar lavage fluid; IPF, idiopathic pulmonary fibrosis; ECM, extracellular matrix. * Serum KL-6 is higher in RAS patients compared to BOS patients and could distinguish between BOS and RAS.

showed that in vitro, fibulin-1C stimulated airway smooth muscle cell proliferation and extracellular matrix production. Similarly, in osteoblasts fibulin-1C was reported to have a negative regulatory effect on proliferation [103]. In this same study, transfecting fibrosarcoma-derived cell lines with fibulin-1D was shown to inhibit their invasive growth potential. Collectively, these studies support the hypothesis that different isoforms of fibulin-1 have different properties and that in the airways fibulin-1C has a specific role in regulating cell proliferation and in production of extracellular matrix, but not in directly controlling the migration of cells.

In vivo, Liu et al. concluded that fibulin-1C deficient mice did not have an increase in collagen deposition following exogenous airway stimuli, in contrast to wild type animals in which collagen accumulation was observed around the airways [108]. In another murine model, deficiency of fibulin-1C inhibited activation of TGF-beta, which further supports the hypothesis that an increase in fibulin-1C is pivotal for the development of fibrosis [109].

Clinically, a study investigating patients with IPF reported that fibulin-1 serum levels were higher in patients with IPF compared to subjects without lung disease. Primary parenchymal fibroblasts from patients with IPF produced significantly more fibulin-1 compared to those from healthy controls. Moreover, serum fibulin-1 levels of IPF patients, at the time of first clinical presentation at a tertiary referral center, predicted disease progression within the first year of follow up [34].

The role of fibulin-1 has not yet been investigated in CLAD but may provide further insight into the fibrotic process in airways.

### 4.3. Pro—C3

The protein fragment pro-C3 is a substitute marker of collagen type III formation [110–112]. Pro-C3 is the N-terminal pro-peptide of collagen type III and is cleaved by proteases in the extracellular space before the mature collagen fibril is incorporated in the extracellular matrix [112,113]. BOS is characterized by increased deposition of type III collagen in the submucosa of the bronchi and this was correlated with poor lung function [26]. Moreover, in obliterative bronchiolitis lesions in a porcine heterotopic bronchial transplantation model, expression of collagen type III mRNA in fibroblast like cells increased in the obliterative lesion when compared to autografts and immunosuppressed allografts [114]. This suggests that collagen type III formation contributes to net deposition of extracellular matrix and thus to fibrosis. In patients with liver fibrosis due to hepatitis or those receiving combined anti-retroviral treatment for HIV, plasma levels of pro-C3 positively correlated with fibrosis and were predictive for disease progression [19,110]. In addition, in IPF patients, levels of pro-C3 were significantly higher in progressive disease compared to stable disease. Interestingly, the ratio of levels of pro-C3 and C3M, a marker for degradation of type III collagen, was lower in stable IPF patients compared to patients with progressive disease [20]. This underlines the idea that progression of fibrosis in IPF is a result of turnover and especially increased deposition of collagen, particularly type III. Since CLAD represents a net deposition of collagen type III, pro-C3 may therefore also serve as a biomarker in CLAD.

### 4.4. Metalloproteinase degraded C-reactive protein

C-reactive protein (CRP) is an acute phase protein that is synthesized by the liver and to some extent deposited in inflamed tissues. Extracellular matrix degradation by MMP-activity generates protein fragments, like CRP degraded by metalloproteinases, that can be found in higher levels in patients with fibrotic lung diseases. Inflammation causes degradation of normal CRP, through proteolytic enzymes, into specific fragments that can provide process-specific information about for example which MMPs are activated [115,116]. In a prospective cohort study, Jenkins et al. compared baseline matrix neoepitope concentrations of patients with IPF with levels in controls, including C-reactive protein degraded by MMP-1/8 (CRPM). A higher baseline CRPM

concentration in serum of patients with IPF was significantly associated with shorter survival in a small cohort, but failed to show significance in a larger cohort [117]. However, a change in CRPM serum levels did affect overall survival hazard ratio. Since inflammation as well as MMP-activity have a substantial role in CLAD, CRPM could also be elevated in CLAD in the early stages of disease.

### 4.5. Antibodies to self-antigens K-α 1 tubulin and Collagen V

Antibodies to k-α 1 tubulin(K-α1T) and collagen type V(Col V) are both associated with development of CLAD [118]. K-α1T is a gap junction protein with mostly intracellular functions [119]. Col V is usually hidden within the structure of collagen type I in the lung tissue extracellular matrix but when exposed can act as an immunogenic ECM protein situated in the perivascular and peribronchiolar tissue [120]. Both neoantigens can be exposed after graft injury, leading to induction of an immune response that is possibly aggravated by loss of peripheral tolerance through suppression of regulatory T-cells by immunosuppression [118]. In particular K-α1T antibodies directly influence the occurrence of airway obliteration through inducing an increase in fibrogenic growth factor expression and fibroproliferation, when these antibodies are bound to alveolar epithelial cells [119].

Higher serum concentrations of anti-K-α1T and anti-col V post LTx were associated with a higher risk to develop BOS in LTx patients. Saini et al. showed that anti-K-α1T levels in serum and BALf were significantly higher in patients with BOS compared to non-BOS when matched for time after transplantation. BALf levels of anti-Col V were also significantly higher in BOS patients compared to non-BOS patients [121]. The occurrence of antibodies directed towards to self-antigens post LTx was shown to be linked to the development of donor specific HLA antibodies in the recipient, potentially representing an interaction between allo- and auto-immunity [118].

Remarkably, antibodies to self-antigens were also found in serum pre-LTX. Antibodies to Col V and K-α1T were found in 70% of LTx patients who had antibodies to self-antigens after transplantation; of whom 73% had persistent antibodies after LTx. Moreover, patients with pre-transplant self-antibodies had a lower BOS free survival compared to LTX patients who did not have pre-transplant antibodies to self-antigens, suggesting that measurement of these antibodies may aid risk prediction for development of BOS after LTx [122].

Antibodies to self-antigens and their role in generating a fibroproliferative response show the complex nature of development of BOS. Currently, it is not known if self-antigen antibodies can serve as a longitudinal biomarker for BOS and more data is needed. Also, further research should elucidate if antibodies to self-antigens can distinguish between BOS and RAS and predict likelihood of disease progression.

### 4.6. Prostaglandin-E2

Prostaglandin-E2 (PGE2), a cyclooxygenase-derived lipid mediator, is a well-known inhibitor of T cells and thus an important immunomodulator [123]. Moreover, PGE2 is known for its anti-fibrotic characteristics, i.e. inhibition of fibroblast migration, collagen synthesis and differentiation of myofibroblast. It is known that deficiency of PGE2 in IPF contributes to reduced fibroblast apoptosis and thus maintain fibroproliferation [124]. Inhaled liposomal PGE2 increased survival in IPF murine models after intratracheal instillation of bleomycin compared to untreated mice [125,126].

PGE2 is produced, among many other cell types, by mesenchymal stromal cells. Mesenchymal stromal cells play a large role in embryonal organogenesis of the lung and are known residents in lung tissue [127]. These cells have the ability to differentiate into myofibroblasts and have a role in the regulation of the micro-environment of the extracellular matrix. These cells are increased in number in BALf preceding onset of BOS [128]. A study in 2012 reported that mesenchymal stromal cells of patients with BOS produced less PGE2 in vitro compared to cells

from non-BOS LTx patients. Mesenchymal stromal cells treated with profibrotic chemokines IL-13/TGF-beta in combination with PGE2 showed less expression of alpha-smooth muscle actin and collagen-1 than cells treated with IL-13/TGF-beta only. Production of PGE2 in BOS mesenchymal stromal cells in culture was also five times lower compared to non-BOS cells and even twenty-two times lower when cells were stimulated with IL-1beta. This suggested failure to upregulate cyclooxygenase-2 (the enzyme responsible for the conversion of arachidonic acid to PGE2, via PGG2 and PGH2 before the generation of the five major prostanoids—PGE2, PGI2, PGD2, PGF2, and thromboxane A2— by their tissue-specific synthases respectively) expression in BOS mesenchymal stromal cells, which was supported by the subsequent western blot analyses investigating cyclooxygenase-2 expression [129]. Furthermore, mesenchymal stromal cells from lung tissue of patients with BOS seem to be resistant to exogenously produced PGE2, for example by epithelium. This was also noticed in IPF previously, possibly caused by differential expression of the receptors for PGE2, with downregulation of *E*-prostanoid receptor 2 in IPF mesenchymal cells [130]. Currently, no data exists on PGE2 as a biomarker for development of BOS, however this should be a target for further research.

## 5. Conclusion

LTx survival is, in about half of the cases, limited by CLAD. Patients with CLAD form a heterogenic population with different disease phenotypes that share a common end stage in the form of fibrosis. Several fibrotic biomarkers have already been investigated in CLAD in different specimens. Serum or sputum seem to be the least invasive methods to acquire samples for regular testing. Present study populations are often small, with unilateral as well as bilateral LTx patients included. This may affect study outcomes since changes in biomarkers could also reflect underlying disease activity in the remaining native lung. In addition, further studies are needed to evaluate if known biomarkers can distinguish between BOS and RAS. Since pathological pathways overlap in different fibrotic diseases, more studies should be established focusing on biomarkers that have already proven their predictive value in detection and progression of fibrotic disease in for example IPF or liver fibrosis. These future studies could meet the urgent need for biomarkers in CLAD for early detection, phenotyping and monitoring disease activity. Moreover, fibrosis biomarkers would give a unique insight in the pathophysiology of this heterogenic disease and could help in developing new treatment strategies. Single biomarkers probably will not adequately represent the different stages and phenotypes in the complex pathophysiological process in CLAD. Several specific biomarkers of fibrosis may predict the clinical course and disease activity in different fibrotic diseases, potentially illustrating contemporaneous processes in different organs. Therefore, a single biomarker may not provide a clear unique description of CLAD. In the future, a panel of biomarkers each encompassing different components of the pathological process of fibrosis will probably best illustrate CLAD and could thus positively impact long term outcomes in LTx. The future for declading the lung will be challenging but promising when we are informed by advances in other fibrotic diseases.

## Funding

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