BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Molecular Profiling in Lung Biopsies of Human Pulmonary Allografts to Predict Chronic Lung Allograft Dysfunction

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Chronic lung allograft dysfunction (CLAD) is the main reason for poor long-term outcome of lung transplantation, with bronchiolitis obliterans (BO) representing the predominant pathological feature. BO is defined as a progressive fibrous obliteration of the small airways, thought to be triggered by a combination of nonimmune bronchial injury and alloimmune and autoimmune mechanisms. Because biopsy samples are too insensitive to reliably detect BO and a decline in lung function test results, which is clinically used to define CLAD, does not detect early stages, there is need for alternative biomarkers for early diagnosis. Herein, we analyzed the cellular composition and differential expression of 45 tissue remodeling-associated genes in transbronchial lung biopsy specimens from two cohorts with 18 patients each: patients who did not develop CLAD within 3 years after transplantation (48 biopsy specimens) and patients rapidly developing CLAD within the first 3 postoperative years (57 biopsy specimens). Integrating the mRNA expression levels of the five most significantly dysregulated genes from the transforming growth factor- β axis (*BMP4*, *IL6*, *MMP1*, *SMAD1*, and *THBS1*) into a score, patient groups could be confidently separated and the outcome predicted ($P < 0.001$). We conclude that overexpression of fibrosis-associated genes may be valuable as a tissue-based molecular biomarker to more accurately diagnose or predict the development of CLAD. (*Am J Pathol* 2015, 185: 3178–3188; <http://dx.doi.org/10.1016/j.ajpath.2015.08.016>)

Chronic lung allograft dysfunction (CLAD) occurs in approximately 50% of patients within 5 years after transplantation and is primarily responsible for the poor long-term outcome of lung transplantation (LTx) compared with other solid organ grafts.¹

CLAD is clinically defined by a sustained decline of respiratory function test results.² This criterion is rather un-specific, though, because alternative causes of functional impairment, such as acute cellular rejection (ACR) or infection, have to be excluded by simultaneous histological and microbiological testing.³ Moreover, lung function testing as a diagnostic parameter is relatively insensitive, because a significant amount of functional lung capacity has to be (irrevocably) lost before becoming clinically apparent. CLAD usually manifests 2 to 3 years after LTx, presenting as a variable, but relentless, deterioration of lung function

with poor response to the few therapeutic modalities available.²

The most prevalent morphological feature in CLAD patients is a progressive fibrous remodeling of the small airways, termed bronchiolitis obliterans (BO).^{4–6} Because of its patchy and discontinuous distribution, BO frequently escapes histological detection by transbronchial biopsies (TBBs), rendering conventional tissue-based diagnostics in CLAD patients insensitive. Still, TBBs are routinely sampled, because they represent, in combination with the clinical assessment of the transplant, the gold standard for monitoring graft function.^{7–9} Their added value lies in the detection and subsequent treatment of clinically

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silent rejection episodes, thus abolishing a potential risk factor for the development and propagation of CLAD.^{10,11}

Because currently available treatment options, like macrolide antibiotics, cytochrome reductase drugs, or switch of immunosuppressive regimens, usually allow stabilization, but not normalization, of pulmonary function, emphasis has been placed on early detection, and potential prevention, of CLAD.^{12,13} Therefore, additional biomarkers culled from blood, lavage, or tissue would be valuable as diagnostic adjuncts to conventional histology and lung function testing. Because the documented success of treating CLAD by macrolide antibiotics at least partially hinges on the manipulation of cytokine signaling in fibrogenesis,¹⁰ unraveling the signal pathways activating myofibroblasts appears promising to uncover potential biomarkers for early CLAD.

Remodeling of the small airways follows a succession of epithelial injury, autoimmune and alloimmune responses, and recruitment, proliferation, and activation of myofibroblasts with excessive matrix production and deposition.^{14–16} Among the signaling pathways regulating myofibroblasts, the equilibrium of extracellular matrix (ECM) turnover is controlled by matrix metalloproteinases (MMPs), their inhibitors (tissue inhibitors of matrix metalloproteinases), and various cytokines, like bone morphogenetic proteins (BMPs), and other members of the transforming growth factor (TGF)- β superfamily.^{17–21} Induction of TGF- β 1 and post-receptor Sma and Mad-related proteins (SMADs) represents a converging pathway that incorporates other profibrotic mediators, such as fibroblast growth factor-2, tumor necrosis factor- α , and endothelin-1.^{15,22,23}

To generate additional biomarkers, TBBs have so far been mostly neglected; predominantly bronchoalveolar lavage (BAL) and blood samples have been analyzed by enzyme-linked immunosorbent assays and radioimmunoassays and cell counts. Elevated numbers of neutrophils, circulating fibrocytes, and helper T cells (with a decrease of T-regulatory cells), as well as increased levels of a multitude of cytokines are associated with the development of CLAD. Although some of these studies were designed prospectively, overall they were hampered by their rather limited number of patients and missing follow-up studies (Table 1).^{7,19,23–45}

Analyzing explanted lung allografts by laser-assisted microdissection, we have previously shown that myofibroblasts in BO lesions are both recipient and donor derived and that airway remodeling in transplanted and non-transplanted patients shares significant molecular traits, such as an up-regulation of pivotal TGF- β 1, BMPs, or plasminogen activator inhibitor 1 (SERPINE1).^{7,22,46} Yet, we also found distinct expression patterns, which set BO lesions in CLAD apart from obliterative airway remodeling in non-transplanted patients. Moreover, we could describe a dysregulated expression of fibrosis-associated genes in morphologically still inconspicuous parenchyma of the lung explant, a field effect only

delimitable on the molecular, not on the histological, level. These markers included, among others, proinflammatory chemokine (C-C motif) ligand 5 (regulated on activation normal T cell expressed and secreted), BMP4, MMP14, and tissue inhibitor of matrix metalloproteinases 1.^{7,22}

These findings open up the possibility of improving the value of tissue-based diagnosis in lung transplants by including molecular data for earlier and more accurate identification of patients at risk of developing BO.

Thus, we evaluated the concept of tissue-based molecular prediction of CLAD in TBB from human pulmonary allografts in two cohorts of clinically well-defined patients, with early-onset CLAD and long-term CLAD-free survival, respectively.

Materials and Methods

Patient Selection and Materials

We retrospectively analyzed lung biopsy specimens sampled in the first year after LTx from two patient cohorts: patients, identified via the biopsy program at Hanover Medical School's (Hanover, Germany), who rapidly developed CLAD (as defined by a continuous >20% decrease in forced expiratory volume in 1 second from baseline) in the first 3 years after LTx (CLAD) and patients with comparable clinicopathological data, but without development of CLAD (within the same observation period; stable). The former group encompassed 18 patients (male/female ratio, 11:7) with a mean age at transplantation of 34.9 (range, 17 to 56) years. Clinical definition and staging of CLAD were performed according to current guidelines.⁴⁷ All pulmonary function tests were performed according to the guidelines of the European Respiratory Society.⁴⁸ A total of 48 lung biopsy specimens (range, 1 to 7 biopsy specimens per patient) sampled 7 to 363 days after LTx were analyzed for this cohort.

The stable cohort consisted of 18 patients (male/female ratio, 10:8) with a mean age at transplantation of 46.7 (range, 19 to 63) years. A total of 57 lung biopsy specimens (range, 2 to 5 biopsy specimens per patient) taken 27 to 364 days after LTx were analyzed for the control group. A mean of 2.5 (range, 1 to 7) biopsy specimens per patient were analyzed for the CLAD group, and 3.2 (range, 2 to 5) for the stable group. End-stage cystic fibrosis, emphysema, and idiopathic pulmonary fibrosis (in descending order) were the leading causes for transplantation, and a combination of cyclosporin A and mycophenolat-mofetil or tacrolimus and mycophenolat-mofetil were the most common immunosuppressants used in both patient groups. Because not all patients had undergone TBB at uniform time intervals (as a result of missed surveillance biopsy specimens because of infection or additional indication-driven biopsy specimens), biopsy (molecular) data were pooled per patient and per group, respectively, and not analyzed longitudinally.

Table 1 Previous Publications on Molecular Analysis of Factors Associated with the Occurrence of BO in Lung-Transplanted Patients

Author (year)	BO/control	Material	Investigated parameters	Method
Devouassoux et al (2001) ²⁴	4/4	TBB	HLA class I antigen, Ki-67, very late antigen-4, integrins	IHC
Reynaud-Gaubert et al (2002) ²⁵	8/13	BAL	IL8, MCP1, CCL5, BAL cell count	ELISA and differential cell count
Meloni et al (2004) ²⁶	8/36	BAL serum (TBB)	MCP1, TGF- β , IFN- γ , IL8 , IL10, IL12 , CCL5, infection, BAL cell count , cellular rejection	ELISA, differential cell count, and TBB (only for rejection)
Taghavi et al (2005) ²⁷	38/34	BAL (TBB)	MMP2, MMP8, MMP9, TIMP1, TIMP2, BAL cell count	ELISA, differential cell count, and TBB (only for rejection)
Hübner et al (2005) ²⁸	8/12	BAL (TBB)	MMP9, TIMP1, BAL cell count	ICC, ELISA, and zymography
Fields et al (2006) ²⁹	20/20	Blood	sCD30	ELISA
Lu et al (2006) ³⁰	11/9	BAL (TBB)	IL1β, CD40LG, TNFSF11, TNFSF9, TNF, IL2, FasL, TNFSF7, LTA, RPL13A, PDGFB, VEGF, BMP6, FGF2, FGF5, ACTB*	RNA microarray (96 targets) and differential cell count
Bharat et al (2007) ³¹	31/31	Blood	IL1β, IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12, IL13, IL15, IL17, eotaxin, CXCL10, MIG, MCP1, MIP-1α, MIP-1β, CCL5, TNF-α, IFN-α, IFN-γ, GM-CSF, IL1α, IL2R	Multiplex bead immunoassay and ELISA
Vanaudenaerde et al (2008) ³²	36/42	BAL	mRNA: IL8, IL17, IL23, TGF-β ; protein: IL1β, IL2, IL6, IL8, IL17, IL23, TGF-β; BAL cell count	RT-PCR, ELISA, and differential cell count
Golocheikine et al (2008) ³³	9/9	Blood	sCD30	ELISA
Neurohr et al (2009) ³⁴	16/47	BAL (TBB)	SLPI, IL8, BAL cell count (% NG)	ELISA and differential cell count
Salama et al (2010) ²³	30/30	Blood/BAL/TBB	Endothelin-1	ELISA (blood) and RT-PCR (TBB)
Bhorade et al (2010) ³⁵	6/14	Blood/BAL	FOXP3⁺ T-cells, CCL17, CCL22	FACS and ELISA
LaPar et al (2011) ³⁶	10/29	Blood	Circulating fibrocyte level	FACS
Jonigk et al (2011) ⁷	24/5	Explanted lung allografts	BMP4, BMPR2, LOX, MMP14, PLAUI, CCL5, THBS1, TIMP1	RNA microarray (45 target genes)
Reilly et al (2011) ³⁷	43/106	BAL	Human neutrophil peptide	ELISA
Wolf et al (2011) ³⁸	48/34	BAL	Clara cell protein, neutrophil defensin 1 and 2, hemoglobin β, calgranulin A, histatin*[†]	Mass spectrometry and
Hall et al (2012) ³⁹	11/8	Blood	IL6, IL8, IL10,	ELISA
Fisichella et al (2013) ⁴⁰	29/76	BAL	IL15, IL17, FGF1, TNF-α, MPO*	Multiplex bead array and ELISA
Shah et al (2013) ⁴¹	60/46	Blood	sRAGE	ELISA
Borthwick et al (2013) ⁴²	26/26	BAL	TNF-α, IL1β, IL8, BAL cell count	ELISA and differential cell count
Jaksch et al (2014) ⁴³	57/85	Blood	YKL40	ELISA
Xu et al (2015) ⁴⁴	10/10	PBMC	miRNAs of the TGF-β and B-cell receptor signal pathway*[†]	RT-PCR
Brugiere et al (2015) ⁴⁵	19/44	BAL/TBB/blood	HLA-G, sHLA-G (TGF-β, IL6, IL8, MCP-1, CXCL10, CCL5, IL17, IL10, GM-CSF, TNF-α, IFN-γ, CXCL9, IL13)[‡]	IHC (HLA-G), ELISA, and Luminex
Heijink et al (2015) ¹⁹	20/20	BAL	MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP12, MMP13, TIMP1, TIMP2	Luminex

Significantly changed markers are highlighted in bold.

*Only significantly changed markers.

†Among others.

‡No analysis between BO/control group reported.

ACTB, β -actin; BAL, bronchoalveolar lavage; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; BO, bronchiolitis obliterans; CCL, C-C chemokine ligand; CD40LG, CD40 ligand; CXCL10, interferon γ -induced protein 10; ECP, eosinophilic cationic protein; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FasL, Fas ligand; FGF1, fibroblast growth factor 1; FOXP3, forkhead box P3; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human leukocyte antigen; ICC, immunocytochemistry; IFN, interferon; IHC, immunohistochemistry; LTA, lymphotoxin- α ; LOX, lysyl oxidase; MCP1, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MIG (CXCL9), monokine induced by γ interferon; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NG, neutrophilic granulocyte; PDGFB, platelet-derived growth factor subunit B; PLAUI, plasminogen activator (urokinase); RPL13A, 60S ribosomal protein L13a; sCD30, soluble CD30; SLPI, secretory leukocyte protease inhibitor; sRAGE, soluble receptor for advanced glycation end product; TBB, transbronchial biopsy; TGF, transforming growth factor; THBS1, thrombospondin-1; TIMP1, tissue inhibitor of matrix metalloproteinases 1; TNF- α , tumor necrosis factor- α ; TNFSF7, TNF ligand superfamily member 7; VEGF, vascular endothelial growth factor; YKL40, chitinase-like glycoprotein.

Sample Preparation

Histopathological Evaluation

The formalin-fixed and paraffin-embedded TBBs were processed as follows: for routine histopathological investigation, serially cut sections were prepared for hematoxylin and eosin, Elastica van Gieson, and periodic acid–Schiff reaction staining. The biopsy specimens were evaluated by two experienced histopathologists (D.J. and F.L.), according to the latest International Society for Heart and Lung Transplantation (ISHLT) classification, with special emphasis on inflammatory changes (see below). The analysis was handled according to the requirements of the local ethics committee (Hanover Medical School's ethics committee vote 2050-2013). We assessed the size of the biopsy particles (scored as number of $\times 100$ and $\times 200$ magnification fields per biopsy) and the percentage of alveolar parenchyma overall. Biopsy specimens that did not fulfill the minimum requisite amount of alveolated lung tissue (minimum of 100 alveoli with pre-capillary and post-capillary blood vessels per high-power field) were excluded from the study.

Grading of Rejection and Airway Inflammation

Grading of rejection and airway inflammation was performed according to the current ISHLT criteria (A0, no acute cellular rejection; A2, mild acute cellular rejection; A3, moderate acute cellular rejection; A4, severe acute cellular rejection; Bx, airway inflammation cannot be assessed; B0, no small airway inflammation; B1R, low-grade small airway inflammation; or B2R, high-grade small airway inflammation). Moreover, we assessed the overall density of the inflammatory infiltrate in bronchi and bronchioli [no inflammation (score, 0), inflammatory cells only delimitable at high magnification ($\times 400$; score, 1), inflammatory cells well recognizable at medium magnification ($\times 200$; score, 2), and high density of the inflammatory infiltrate well visible even at low magnification ($\times 100$; score, 3)].² For each patient, the maximum ISHLT A and B scores were tabulated. ACR was summarized to not present (A0) and present (A1 or higher), as was small airway inflammation (B0 and B1R or higher, respectively). The resulting data were compared by Fisher's exact test.

Alveolar Macrophages

Furthermore, we graded the amount of intra-alveolar macrophages semiquantitatively [no macrophages ($\times 400$; score, 0), sparse macrophages only delimitable at high magnification ($\times 400$; score, 1), macrophages well recognizable at medium magnification ($\times 200$; score, 2), and high density of macrophages with a desquamative interstitial pneumonia-like (DIP) aspect, well visible even at low magnification ($\times 100$; score, 3)]. Alternatively, we also performed a direct count on the basis of a CD68 staining (PGM-1; Dako, Hamburg, Germany): after identification and registration of macrophage hot spots, we counted the number of macrophages per sectioned alveolus in 20 adjacent alveoli within the main hot spots.

RNA Extraction and cDNA Generation

After histomorphological and immunohistochemical evaluation, the whole remaining biopsy tissue was cut into sections (10 μm thick) and suspended overnight in a proteinase K digestion solution. RNA was isolated using phenol-chloroform extraction, followed by ethanol precipitation via our established protocol. RNA (10 μL) was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), following the manufacturer's guidelines.^{7,49}

Low-Density Arrays

To analyze the mRNA expression of 45 tissue remodeling and myofibroblast activation-associated genes and three reference genes (Supplemental Table S1), the cDNA was evaluated by real-time PCR (7900HT Fast Real-Time PCR system) using TaqMan-based low-density/high-throughput arrays. Target genes were selected on the basis of our previous analyses of BO lesions, laser microdissected from explanted human pulmonary allografts.^{7,22} The low-density arrays contained primers with an amplicon size of 100 bp or less and were spotted eightfold into a 384-well plate, which facilitated synchronous analysis of eight samples per PCR run. Accordingly, the low-density arrays were recorded using 7900 HT SDS software version 2.3 Rev C (Applied Biosystems) to calculate the CT values.⁷

Statistical Analysis

Gene expression values were converted to relative expression (using the ΔCT method with polymerase-2 α as endogenous reference and relative expression calculated as $2^{-\Delta\text{CT}}$). Relative expression values of genes not detected in RT-PCR were set to 0. Gene expression values were compared between groups with the Wilcoxon test. All tests were regarded as exploratory, with no correction for multiple hypothesis testing.

Scoring of Gene Expression in Lung Biopsy Specimens

The variable importance for the mRNA expression levels of 45 remodeling-associated genes was estimated by random forest classification analysis [implemented by the R package random forest version 4.6-7 (<http://www.r-project.org>)].⁵⁰ The five highest ranked genes were selected for further analysis. Three target genes (*IL4*, *IL13*, and *IL17A*) with low expression in the CLAD and the stable patient group were discarded beforehand. To simplify further testing, the 95th percentile of the relative expression values in controls was used as a cutoff. If the relative expression of the gene of interest was higher than the cutoff, 1 point; otherwise, 0 points were awarded. Finally, all points for the established five genes of interest (see Results) were added together, resulting in a score with a minimum of 0 and a maximum of 5 points. All calculations were performed with R version 3.1 (<http://www.r-project.org>) on an IBM-compatible personal computer running Windows 7 (32 bit). Up-regulated genes were mapped onto pathways and biological functions using Ingenuity

Figure 1 Correlation of bronchial and bronchiolar wall inflammatory infiltration (score, 0 to 3) with the International Society for Heart and Lung Transplantation B scores in the chronic lung allograft dysfunction (CLAD; open circles) and the stable groups (open triangles). B0, no small airway inflammation; B1R, low-grade small airway inflammation; B2R, high-grade small airway inflammation; Bx, airway inflammation cannot be assessed; NA, not available.

pathway analysis software version 24718999 (Qiagen, Venlo, the Netherlands).

Immunohistochemistry

To assess the complementary protein expression, immunohistochemical staining for the five highest ranked genes was performed, following our established protocol.⁷

Analysis of Macrophage Counts

Count data were analyzed with a negative binomial generalized mixed model [R-package lme4 1.1-7 (<http://www.r-project.org>)], treating the patient as a random effect to account for overdispersion and multiple observations (biopsy specimens) per patient. Furthermore, the presence of macrophage hotspots [defined as clusters of macrophages delimitable at low-power magnification ($\times 100$)] was recorded. A binomial generalized mixed model (lme4) was used to test for differences in hotspot presence.⁵⁰

Results

We retrospectively analyzed lung biopsy specimens sampled from two patient cohorts: long-term survivors, who did not develop CLAD in the first 3 years after LTx (stable; 18 patients, 57 biopsy specimens), and patients who rapidly developed CLAD within the same observation period (18 patients, 48 biopsy specimens).

ISHLT Scores

ISHLT A Scores

Overall, more patients in the stable group ($n = 10$) experienced an episode of ACR (A1 or above), compared

with the CLAD group ($n = 4$). However, this difference was not statistically significant [odds ratio (OR), 3.31; 95% CI, 0.65–20.14; $P = 0.158$].

ISHLT B Scores

There were significantly more stable patients with low-grade small airway inflammation (ISHLT-B) than CLAD patients (OR, 35.91; 95% CI, 3.56–1919; $P < 0.001$).

ISHLT C Scores

No biopsy of our CLAD group contained part of a BO lesion (all C0).

Airway Inflammatory Infiltrates

Paralleling the findings for ISHLT B scores, biopsy specimens of the stable group showed higher scores for bronchial/bronchiolar wall inflammatory infiltrates (mean score, 0.82; range, 0 to 2), compared with the CLAD group (mean score, 0.37; range, 0 to 3). The infiltrate scores correlate positively with the ISHLT B scores ($\tau = 0.86$, $P < 0.0001$) (Figure 1).

Intra-Alveolar Macrophages

Alveolar macrophage (AM) counts were 59% (95% CI, 44%–80%) lower in biopsy specimens of CLAD patients, compared with stable patients ($P < 0.001$). Mean values were 60.0 AMs/20 alveolar spaces (interquartile range, 27.0 to 86.5) for the CLAD group and 89.7 AMs/20 alveolar spaces (interquartile range, 57.0 to 115.0) for the stable group (Figures 2 and 3).

These counts, on the basis of immunohistochemical marking of AM, showed a moderate correlation with the macrophage score by semiquantitative estimation (score, 0 to 3; $r = 0.38$, $P < 0.001$) after the removal of outliers ($r = 0.29$, $P < 0.01$). AM hotspots could be detected more frequently in biopsy specimens of stable patients (59.7%) than in CLAD patients (37.5%), showing only a trend toward statistical significance ($P = 0.058$). Within the same patients, there were no relevant changes in macrophage density over time or in between the available TBB.

Figure 2 Alveolar macrophages (AMs) per 20 alveoli in the chronic lung allograft dysfunction (CLAD) and the stable groups, shown per individual biopsy (A) and per patient average (B). * $P < 0.05$, ** $P < 0.01$.

Figure 3 Alveolar macrophages stained by hematoxylin and eosin (A and B) and immunohistochemically against CD68⁺ (clone PGM-1; C and D) in transbronchial lung biopsy specimens of patients who rapidly developed chronic lung allograft dysfunction (CLAD; A and C) and patients who did not develop CLAD in the first 3 years after lung transplantation (stable; B and D). Note the prominent intra-alveolar content of macrophages in the stable group compared with the CLAD group. Boxed areas in insets indicate areas of higher magnification in the main panel. Original magnification, $\times 400$ (A–D, main images); $\times 100$ (A–D, insets).

Gene Expression in CLAD and Stable Groups: Random Forest Analysis

In TBB from the CLAD group, we found a significant up-regulation of 22 genes, compared with the stable group (Supplemental Table S1).

Of the differentially expressed genes outlined above, the five statistically (and biologically) most important variables for separation of the CLAD and stable groups, as determined by random forest analysis, were *BMP4*, *IL6*, *MMP1*, *SMAD1*, and *THBS1*. Relative expression values for all their gene products were significantly higher in biopsy specimens of CLAD than stable patients (Figures 4 and 5).

Classification of Lung Biopsy Specimens

The relative cutoff values (95th percentile of relative expression in stable patients) were 0.17 for *IL6*, 0.02 for *MMP1*, 0.65 for *SMAD1*, 4.78 for *THBS1*, and 0.69 for

BMP4. Awarded points were summed up in every biopsy, resulting in the CLAD score.

The maximum points scored in CLAD biopsy specimens were four of five possible points and two of five maximum points in stable patients. The score was then condensed to 0, 1, and 2 or higher (2+), because values of >2 did not aid in discriminating the CLAD group from the stable group. Of biopsy specimens, 64.6% from the CLAD group were scored as 2 or higher (2+); 16.7% received a score of 1; and 18.8%, 0. In contrast, only 1.8% of the stable patients' biopsy specimens were scored as 2+; 19.3%, 1; and 78.9%, 0 (Figure 6).

Analysis of biopsy specimens sampled after day 220 after LTx did not change the grouping of the patients, and no patient developed a positive CLAD score after this time point. In the CLAD group, 62% of all biopsy specimens showed at least a score of 2, with only three patients (15%) displaying lower scores in all biopsy specimens analyzed. In the stable group, only a single biopsy (2%) revealed a score of 2 or higher, with biopsy specimens of nine patients (50%) showing a score of 0 and 8 patients (44%) a score of 1. CLAD scores were not evenly distributed among patient groups ($P < 0.001$, $\chi^2 = 22.149$) with more 2+ (high) scored biopsy specimens in the CLAD group.

Immunohistochemical staining for *BMP4*, *IL6*, *MMP1*, *SMAD1*, and *THBS1* concurred with the mRNA expression (Supplemental Figure S1).

AM count (per 20 alveoli) showed a moderate negative correlation with the raw CLAD score [0 to 5 points; Pearson's $r = -0.36$ (range, -0.53 to -0.18); $P < 0.001$].

Discussion

Because of the lack of sensitivity of conventional morphology in TBBs for CLAD, we and others started looking for means to supplement conventional histological analysis of invasively sampled specimens, which would allow an earlier and more specific diagnosis of CLAD, making earlier treatment with potentially better outcome possible.^{7,19} In the present study, we wanted to validate the predictive value of a set of molecular markers for CLAD, as previously established by us in lung explants, in a retrospective analysis of TBBs from LTx patients.^{7,22}

Figure 4 Relative mRNA expression levels for IL-6, matrix metalloproteinase 1 (MMP1), Sma and Mad-related protein 1 (SMAD1), thrombospondin 1 (THBS1), and bone morphogenetic protein 4 (BMP4) in lung biopsy specimens of rapid-onset chronic lung allograft dysfunction (CLAD) and the stable groups. ** $P < 0.01$, *** $P < 0.001$.

Figure 5 Visualization of pathways and functions affected via chronic lung allograft dysfunction (CLAD)—associated and up-regulated genes. Genes that were found to be up-regulated in biopsy specimens of CLAD patients were mapped onto pathways and biological functions (orange). Of these, the five most important for separation of the rapid-onset CLAD and the stable groups were *BMP4*, *IL6*, *MMP1*, *SMAD1*, and *THBS1* (marked and highlighted in red). *ATG16L1*, autophagy-related protein 16-1; *BMP*, bone morphogenetic protein; *BMPR2*, bone morphogenetic protein receptor type II; *CCL5*, chemokine (C-C motif) ligand 5; *CD36*, fatty acid translocase; *CD47*, integrin associated protein; *COL3A1*, collagen, type III, alpha 1; *CXCL8*, IL-8; *CXCL12*, CXC-motiv-chemokine 12; *HNRNP1*, heterogeneous nuclear ribonucleoprotein A1; *LOX*, lipoxigenase; *MMP*, matrix metalloproteinase; *PLAT*, plasminogen activator, tissue; *PLAUR*, plasminogen activator, urokinase receptor; *PLOD2*, procollagen-lysine; *RUVBL2*, RuvB-like AAA ATPase 2; *SDC*, syndecan; *Serpine1*, plasminogen activator inhibitor-1; *SH3GLB1*, SH3-domain GRB2-like endophilin B1; *Terf2*, telomeric repeat-binding factor 2; *TGF*, transforming growth factor; *THBS1*, thrombospondin 1; *VHL*, Von Hippel-Lindau tumor suppressor.

Conventional histopathological and ISHLT criteria (ACR, presence and density of airway inflammation, and remodeling) revealed no significant differences between the two groups, underlining their lack of sensitivity regarding CLAD. Nevertheless, airway inflammation and recurrent episodes of ACR have been described as risk factors for the future development of CLAD, a correlation we also did not find in our groups. This is most likely because of our limited observation period and the fact that at Hanover Medical School, unlike other transplant centers, clinically silent rejection episodes are diagnosed because of the established surveillance biopsy program and are immediately and aggressively treated.^{47,51–54}

Regarding the potential use of cytokine profiles and expression data to predict patient outcome after LTx, several groups have reported, in >20 publications, data concerning a multitude of factors involved in airway remodeling (Table 1). However, so far, no biomarker pattern has been introduced into routine clinical practice.^{7,19,23–45}

In our work, we found an up-regulation of a larger number of mediators governing different fields of function, such as epithelial-mesenchymal transition, vascular remodeling, hemostasis, and macrophage recruitment, and activation in biopsy specimens from patients of the CLAD group (Supplemental Table S1). Particularly, so far, mostly unreported gene products associated with the BMP–TGF- β axis and particularly myofibroblast activation showed a differential expression between the two patient groups and emerged as promising diagnostic markers to identify patients at risk for developing CLAD. These potential biomarkers included *BMP4*, *IL6*, *MMP1*, *SMAD1*, and *THBS1* (Figure 5).

Because most comparable approaches of molecular testing focused on BAL samples, parallel testing of biopsy specimens

and complementary lavages would have been desirable. Unfortunately, biobanked BAL specimens adequately preserved for reliable molecular testing were not available for these retrospective cohorts.

A continuous increase of proinflammatory cytokines has been put forward in recent publications as preceding and predicting CLAD.^{26,55,56}

The central role of proinflammatory *IL6* in tissue remodeling, (autoimmune) pulmonary disease, and pulmonary vascular aging has been recognized for some time.^{57–59} Its important role in LTx failure has been recognized more recently: *IL6* elevation in blood and BAL of recipients and even in the graft before LTx has been shown to be associated with the

Figure 6 Classification of transbronchial biopsy specimens according to the chronic lung allograft dysfunction (CLAD) score (points awarded for the increased expression of *IL-6*, matrix metalloproteinase 1, *Sma* and *Mad*-related protein 1, *thrombospondin 1*, and *bone morphogenetic protein 4* were summed up in every biopsy specimen of every patient, resulting in the respective CLAD score of the biopsy specimens). The point score was condensed to 0, 1, and 2 or higher (2+), because values of >2 did not aid in discriminating the rapid-onset CLAD (dark gray bars) and the stable (light gray bars) groups. Number of biopsy specimens is given.

occurrence of more frequent rejection episodes, earlier manifestation of BO syndrome, and restrictive allograft syndrome and a greater chance of death.^{31,39} This outcome seems to be the sequelae of an exaggerated inflammatory response with consecutive amplified alloimmunity, type 17 helper T-cell activation, proliferation, and induction of TGF- β -dependent airway remodeling.^{31,55,60} These findings, especially regarding the overexpression of IL6, match our own.

Likewise, we found THBS1, a member of the thrombospondin family of multifunctional proteins, a strong differentiator between the CLAD and stable groups and describe its potential role and diagnostic value in CLAD for the first time. In health and disease, THBS1 is secreted into the ECM and interacts with at least a dozen cellular receptors, among them syndecans, integrins, integrin-associated protein, and fatty acid translocase (CD36), regulating cell migration and ECM remodeling.⁶¹ More recent findings suggest an activating THBS1-TGF- β axis in fibrosing and inflammatory lesions in visceral organs, emphasizing its potential use as a biomarker in fibrosing lung diseases.⁶¹ This corresponds to *in vitro* and *in vivo* findings that THBS1 activates latent TGF- β secreted by AMs via interaction with plasmin and CD36. It could be shown that THBS1 overexpression correlated with the onset and exacerbation of idiopathic interstitial pneumonias, particularly with a decrease in vital capacity.^{61–64}

Of the other mediators we found strongly associated with BO, BMP4 is of particular interest. The role of the BMPs, particularly BMP4, in lung disease is far from clear: an MMP-mediated effect of BMP4 on soft tissue hemostasis in humans, direct inhibition of epithelial cell proliferation and differentiation, and induction and repression of ECM maturation have all been described.^{20,21} In experimental systemic application in chronic allograft nephropathy, BMP4 and BMP7 inhibited the transformation of fibroblasts to activated myofibroblasts, consecutive ECM deposition, and smooth muscle metaplasia via the activin receptor-like kinase 1 (ALK1)/SMAD1/5 pathway.^{20,21,65,66} So, the exact role of BMP4 and BMP7 in tissue remodeling in adults remains unclear, particularly in the lungs, and we are the first to address it in regard to diagnosing CLAD. Most likely, BMP4, produced by the remnant respiratory cells, acts profibrotic or antifibrotic in a concentration-dependent manner via the TGF- β pathway, with subsequent phosphorylation of cytosolic SMADs by a BMP receptor kinase.²¹ Likewise, BMP7 is known to counteract TGF- β -induced epithelial-mesenchymal transition and facilitate epithelial regeneration in visceral organs. Somewhat comparable, SMAD1 can also act in a profibrotic and antifibrotic manner: SMAD1, and potentially also SMAD5 and SMAD8, are directly recruited by Alk1, an alternative TGF- β receptor, to drive the expression of a subset of profibrotic genes, via the Alk1/SMAD1 axis.

Of special interest in lung remodeling is the transition of fibroblasts to myofibroblasts, which is paralleled by the expression of smooth muscle actin and smooth muscle

myosin.¹⁶ In this way, the SMAD1/5/8 complex is a common downstream mediator of TGF- β signaling in primary and secondary fibrosing pulmonary diseases, such as idiopathic pulmonary fibrosis or pulmonary manifestation of scleroderma.^{65,67,68} Likewise, it has been shown *in vitro* that some of the antifibrotic effects of the tyrosine kinase inhibitor imatinib (Glivec; Novartis, Basel, Switzerland) are mediated by inhibition of SMAD1, with subsequent repression of TGF- β -mediated connective tissue growth factor and collagen type I and formation of ECM.⁶⁵

All of the mediators listed above regulate TGF- β , the lynchpin of tissue remodeling in the airways. Of the downstream effectors, we found MMP1 to be an indicator of impending BO. MMP1 is able to break down fibrillar collagens I, II, and III.^{14,17} Correspondingly, increased expression of MMP1 in chronic obstructive pulmonary disease corresponds to the degree of disintegration of alveolar septa and the loss of vital capacity.¹⁴ However, tissue degradation by MMPs also unveils autoantigens, such as K- α 1-tubulin and type-V collagen, which, in a vicious circle, trigger and strengthen an autoimmune response, initially leading to MMP activation.^{11,12,69} Furthermore, it has also been demonstrated that the degradation of structural cells and ECM liberates and activates dormant signaling molecules like pro-TGF- α and other members of the epidermal growth factor family. These, in a positive feedback loop, induce the TGF- β cascade and promote further tissue breakdown.^{21,65,70}

Accordingly, down-regulation of MMP1 is an important step in physiological wound healing, whereas lingering wounds are characterized by an uncontested overexpression.^{42,64} Interestingly, MMP1 has also been reported intracellularly and is thought to be vital in cell division, migration, differentiation, and, ultimately, apoptosis.^{14,17} This might explain the prominent up-regulation of MMP1 in progressive fibrosing diseases like idiopathic pulmonary fibrosis.⁷¹ Systematic analyses of the role of different MMPs in CLAD development in human patients have, for the most part, been performed in BAL specimens. In these, especially MMP9 has come into the center of interest, whereas an overexpression of MMP1 could not be found.^{19,27} The fact that we can describe an up-regulation of MMP1 in TBB of CLAD patients indicates different compartments sampled by BAL or TBB, and is an argument for tissue-based molecular testing in LTx patients.

In a systematic assessment of the inflammatory cells in the analyzed biopsy specimens, we found significantly more intra-alveolar macrophages in the stable group compared with the CLAD group. This principally agrees with the findings of others, who reported a decrease in macrophages in the BAL of LTx patients with manifested BO syndrome.^{27,40} In BAL samples, this was so far attributed to the relative increase in neutrophils and lymphocytes as the cytological hallmark of a shift toward a proinflammatory and profibrogenic microenvironment in (early) CLAD.^{63,68} We found a distinct up-regulation of IL6 and MMP1 only

in the CLAD group, a signature associated with classically activated M1 macrophages.^{63,64} Conversely, we speculate that the low levels of these proinflammatory cytokines and enzymes in the stable group, with abundant intra-alveolar macrophages present at the same time, might point toward an alternate, and potentially beneficial, state of activation. One of these is the M2 subtype, which is associated with immunomodulation, maintenance of self-tolerance, and tissue hemostasis as well as wound healing and ECM reconstitution.^{63,64,72} The M1/M2 phenotypes are dynamic states, exhibited in response to exogenous or endogenous stimuli, such as TGF- β and BMP4 and BMP7, and macrophage populations can revert from one to the other given a change of microenvironment.^{64,73} However, under the immunosuppressive regimen after LTx, it is known that proinflammatory IL expression is reduced in macrophages, which might lead to an inactive state, associated with inactivation of autoreactive or alloreactive T cells.⁵⁸ This represents a crucial step in the decrease of cellular rejection by induction of tolerance against allo-antigens and (physiologically sequestered) auto-antigens.⁷⁴ In our study, the alveolar concentration of macrophages remained stable over time, in individual patients and within patient groups and correlated inversely with the CLAD score, in contrast to all other inflammatory cells, characterized by the ISHLT score. Accordingly, the differences in concentration of intra-alveolar macrophages might reflect the levels of local inflammatory mediators in the graft in response to continuous injurious stimuli. Nonetheless, one would rather expect increased accumulation of macrophages in the transplant, because abundant macrophages are found in the inflammation-rich early BO lesions, formerly designated as active in the 1995 ISHLT formulation; these show predominantly an M1 phenotype. So, the role of AMs in the development or prevention of CLAD should be investigated more closely. Particularly the influence of different macrophage subpopulations on graft fate should be assessed by functional experiments and analysis of the cytokine micromilieu.

We conclude that further investigations on the potential use of molecular biomarkers for CLAD in TBB are warranted. On the basis of our results and of others, groups of genes from the TGF- β axis, particularly *BMP4*, *IL6*, *MMP1*, *SMAD1*, and *THBS1*, present themselves as promising targets and may act as predictive markers, particularly for early events contributing to the subsequent development of CLAD. Further studies should follow a prospective design and analyze TBB and complementary BAL samples at the same time.

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Supplemental Data

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