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The Journal of Heart and Lung Transplantation

http://www.jhltonline.org

Heterogeneity of chronic lung allograft dysfunction: Insights from protein expression in broncho alveolar lavage

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KEYWORDS:
bronchiolitis obliterans syndrome;
chronic lung allograft dysfunction;
lung transplantation;
neutrophilic reversible allograft dysfunction;
bronchoalveolar lavage;
lung rejection

BACKGROUND: Chronic lung allograft dysfunction (CLAD) remains a major risk factor for death after lung transplantation. Previous data suggested that within CLAD at least 2 phenotypes are present: a neutrophilic type (nCLAD or neutrophilic reversible allograft dysfunction [NRAD]), reversible with azithromycin therapy, vs a low neutrophilic type, non-responsive to azithromycin (fibrotic bronchiolitis obliterans syndrome [fBOS]). We aimed to further characterize this dichotomy by measuring multiple proteins in the bronchoalveolar lavage (BAL) fluid of 28 lung recipients.

METHODS: Patients were retrospectively subdivided by the absence or presence of CLAD and subsequently by their response to azithromycin, resulting in 3 groups: 10 stable, 9 responsive (nCLAD/NRAD), and 9 non-responsive (fBOS). Enzyme-linked immunosorbent assay was used to measure 32 different proteins.

RESULTS: Protein variations were predominantly present in the nCLAD/NRAD group, whereas no differences were observed in the fBOS group compared with control. MCP-1 ($p < 0.01$), RANTES ($p < 0.05$), IL-1β ($p < 0.01$), IL-8 ($p < 0.01$), TIMP-1 ($p < 0.01$), MMP-8 ($p < 0.01$), MMP-9 ($p < 0.01$), HGF ($p < 0.001$), MPO ($p < 0.01$), and bile acid ($p < 0.05$) concentrations were upregulated in nCLAD/NRAD compared with fBOS, whereas PDGF-AA ($p < 0.05$) was downregulated.

CONCLUSIONS: These data provide further evidence that within CLAD there is a heterogeneity of phenotypes with different mechanisms involved. Further investigation is warranted to unravel the pathophysiology of both phenotypes.

J Heart Lung Transplant 2011;30:667–73

Survival after lung transplantation (LTx) is hampered by bronchiolitis obliterans syndrome (BOS), defined as an irreversible decline in forced expiratory volume in 1 second (FEV_1) by at least 20% compared with the best post-operative values, in the absence of other causes.[1] It affects 45% to 50% of LTx patients after 5 years and accounts for 30% of late mortality.[2] We and others have shown that LTx patients who develop a progressive loss in FEV_1, with presence of bronchoalveolar lavage (BAL) neutrophilia >15% to 20%, may respond to azithromycin treatment.[3–5] This evolution is no longer consistent with the definition of BOS, and as a consequence, the term chronic lung allograft dysfunction (CLAD) was introduced to further distinguish reversible and non-reversible causes of FEV_1 decline.[6] The non-reversible phenotype remains consistent with BOS,

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1053-2498/$ -see front matter
doi:10.1016/j.healun.2010.12.008

Abbreviations for Factors Analyzed in Bronchoalveolar Fluid	
Cyclooxygenase 2	COX2
Epidermal growth factor	EGF
Basic fibroblast growth factor	FGFb
Granulocyte colony-stimulating factor	G-CSF
Granulocyte macrophage colony-stimulating factor	GM-SCF
Growth-regulated oncogene-α	GRO-α
Hepatocyte growth factor	HGF
Interferon-γ	IFN-γ
Interleukin	IL
Monocyte chemotactic protein-1	MCP-1
Metalloproteinase	MMP
Myeloperoxidase	MPO
Neutrophilic chronic lung allograft dysfunction	
Neutrophilic reversible allograft dysfunction	
Platelet derived growth factor-AA	PDGF-AA
Placental growth factor	PlGF
Regulated on activation normal T cell-expressed and secreted	RANTES
Receptor for advanced glycation end products	RAGE
Stromal cell-derived factor-1	SDF-1
Surfactant protein-C	SP-C
Transforming growth factor-β1	TGF-β1
Tissue inhibitor of metalloproteinase 1	TIMP-1
Tumor necrosis factor-α	TNF-α

whereas the reversible phenotype was renamed as neutrophilic reversible allograft dysfunction (NRAD)[4] or as neutrophilic CLAD (nCLAD).

The existence of nCLAD/NRAD was recently confirmed in a randomized placebo-controlled trial in which addition of azithromycin at discharge after LTx prevented the development of nCLAD/NRAD.[7] This possible improvement or restoration of FEV_1 with azithromycin in these nCLAD/NRAD patients is a contradictory finding. Consequently, we aimed to characterize both CLAD phenotypes by looking at BAL proteins reflecting inflammation, oxidative stress, angiogenesis, matrix remodeling, fibrosis, and reflux. Improved understanding of the mechanisms could pave the way for a better outcome after LTx.

Materials and methods

This study was approved by the hospital's local Ethics Committee.

Patient characteristics

Patients were included in the present study if they had a progressive decline in pulmonary function of at least 20% and had a bronchoscopy with BAL and transbronchial biopsy upon CLAD diagnosis. Acute rejection, overt infection, and other causes for chronic decline in FEV_1 were excluded. Moreover, around that time, azithromycin had to be started, as displayed in Figure 1. CLAD patients were retrospectively sub-divided in 2 sub-groups to be confident about their phenotype according to the following criteria: FEV_1 increase of at least 10% at 3 to 6 months after initiating azithromycin (nCLAD/NRAD), whereas fBOS was defined as a further decrease in FEV_1, despite azithromycin treatment. A control group (free of CLAD) was matched according to the post-operative time of BAL sampling in the CLAD group (Figure 2).

Lung function, BAL, and bronchoscopy

Spirometry was performed according to international guidelines.[8] For BAL, 2 aliquots of sterile saline (50 mL) were instilled in the right middle lobe or lingula. The returned fractions were pooled and processed for cell counting and supernatant collection for protein measurement as previously described.[5] Biopsy specimens were taken after BAL, examined and graded by a pathologist skilled in LTx according to the international guidelines.[1]

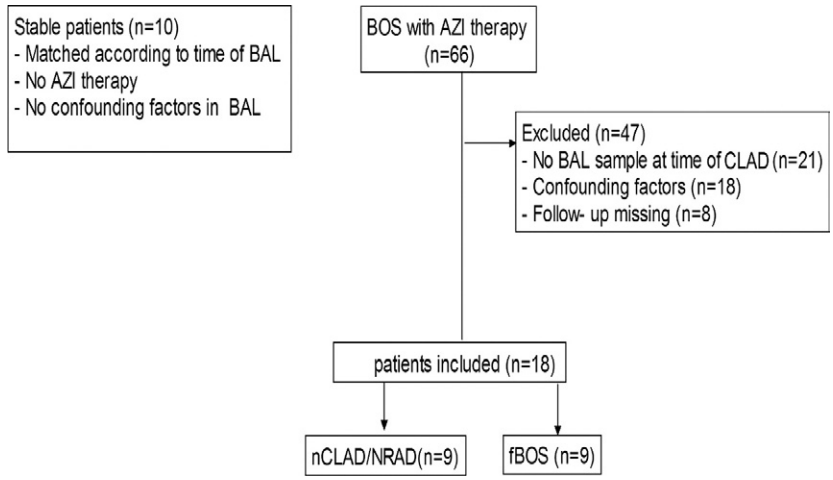

Figure 1 Patient selection criteria. Stable patients were chosen according to the post-operative timing of bronchoalveolar lavage (BAL), the absence of azithromycin (AZI), and the absence of confounding factors in their BAL fluid that could influence the results (acute events, high C-reactive protein, colonization, cytomegalovirus, or other infection at the moment of BAL). The study included 18 patients. Patients with chronic lung allograft dysfunction (CLAD) were excluded due to multiple reasons: for 21 patients there was no BAL sample at diagnosis of CLAD, 18 had a confounding factor in their BAL, and 8 did not have sufficient follow-up. fBOS, fibrotic bronchiolitis obliterans syndrome; nCLAD, neutrophilic CLAD; NRAD, neutrophilic reversible allograft dysfunction.

Evaluation for gastroesophageal reflux and bile acid measurement

The pH impedance measurement was performed as previously described to measure total number of reflux events, bolus exposure, and acid exposure.[9] When no such assessment was available, gastroscopy data were used to determine whether or not the patient was suffering from reflux. Bile acids were quantified in 3-μl of undiluted BAL fluid in duplicate with a commercially available enzymatic assay (detection limit 0.2 μmol/liter; Bioquant, San Diego, Calif). None of the patients had undergone Nissen fundoplication.

BAL protein measurement

Analysis of human protein expression was measured in BAL supernatant. Selected proteins (COX2, osteopontin, RANTES, RAGE, GROα, MCP-1, TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-23, MPO, SP-C, FGFb, VEGF, PlGF, HGF, PDGF-AA, TIMP-1, EGF, TGF-β1, MMP-8, MMP-9, GM-CSF, G-SCF, fibronectin, and SDF-1 [see Abbreviations]) were measured by custom multiplex SearchLight Assay System (Aushon, Billerica, MA). As a pilot experiment, 400 μl of 12 selected samples (4 of each group) were shipped on dry ice. All samples were analyzed in duplicate.

Based on the results of this pilot experiment, we analyzed the following 19 proteins in 28 BAL samples, comprising 9 nCLAD/NRAD, 9 fBOS, and 10 stable patients: RANTES, RAGE, GROα, MCP-1, TNF-α, IL-1β, IL-8, MPO, SP-C, FGFb, VEGF, PlGF, HGF, PDGF-AA, TIMP-1, TGF- β1, MMP-8, MMP-9, and fibronectin. Results are discussed when $p < 0.05$. IL-6, osteopontin, GM-CSF, G-SCF, and EGF were also determined in the pilot experiment but were not further investigated due to lack of differences between the groups. Levels of IFN-γ, IL-4, IL-10, IL-12p40, IL-17A, IL-23, SDF-1, and COX-2 were systematically under the detection limit and are therefore not reported. If concentrations of other proteins were under the detection limit in some samples only, a value of 50% of the detection limit was accorded.

Table 1 Patient Characteristics

Variable	Stable ($n = 10$) Mean (IQR) or No. (%)	CLAD ($n = 18$) Mean (IQR) or No. (%)	nCLAD/nRAD ($n = 9$) Mean (IQR) or No. (%)	fBOS ($n = 9$) Mean (IQR) or No. (%)	p-value
Age at transplant, years	43 (32–54)	50 (31–56)	52 (49–59)	35 (29–52)	0.14
Male sex	6 (60)	8 (44)	4 (44)	4 (44)	0.78
Type of Tx					0.31
Double lung	9 (90)	13 (72)	6 (67)	7 (78)	
Single lung	0 (0)	4 (22)	3 (33)	1 (11)	
Heart-lung	1 (10)	1 (6)	0 (0)	1 (11)	
Ischemia, hours	6.7 (6.1–7.3)	5.5 (4.5–6.7)[a]	5.1 (4.3–6.0)[b]	6.5 (4.9–7.3)	0.009
CMV match	4 (40)	12 (67)	5 (56)	7 (78)	0.25
Pre-Tx diagnosis					0.21
Emphysema	4 (40)	8 (44)	7 (78)	1 (11)	
Fibrosis/sarcoidosis	3 (30)	5 (28)	1 (11)	4 (44)	
PAH	1 (10)	1 (6)	0 (0)	1 (11)	
Cystic fibrosis	2 (20)	4 (22)	1 (11)	3 (33)	
Immunosuppressive therapy					
Steroids/none	10/0	17/1	8/1	9/0	0.68
FK/CSA	9/1	15/3	8/1	7/2	0.78
AZA/MMF/none	8/0/2	16/2/0	8/1/0	8/1/0	0.55
GERD					0.69
No assessment	0 (0)	3 (17)	1 (11)	2 (22)	
Gastroscopy	1 (10)	2 (11)	1 (11)	1 (11)	
pH-impedance measurement	9 (90)	13 (72)	7 (78)	6 (67)	
GER on gastroscopy	0	0	0	0	
GER on pH-impedance					
Total number of events	52 (30–70)	34 (23–91)	26 (16–51)	74 (27–109)	0.12
Bolus exposure (<1.4), %	1.3 (0.5–2.2)	1.3 (0.3–4.7)	0.4 (0.2–3.1)	2.1 (0.5–5.5)	0.39
Acid exposure (<4.2), %	2.1 (0.9–7.1)	2.2 (0.6–6.9)	1.9 (0.2–2.6)	4.4 (0.7–10.3)	0.95
Timing of BAL, post-op mon	18.1 (17.8–18.5)	19.7 (10.3–47.7)	15.5 (6.0–36.9)	34.4 (17.2–48.3)	0.07

α1ATD, α1 antitrypsin deficiency; AZA, azathioprine; BAL, bronchoalveolar lavage; CLAD, chronic lung allograft dysfunction; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CSA, cyclosporine; fBOS, fibrotic bronchiolitis obliterans syndrome; FK, tacrolimus; GERD, gastrointestinal reflux disease; IQR, interquartile range; LTx, lung transplantation; MMF, mycophenolate mofetil; NA, not applicable; nCLAD, neutrophilic CLAD; NRAD, neutrophilic reversible allograft dysfunction; PAH, pulmonary arterial hypertension; Tx, transplantation.

The difference between the stable, nCLAD/NRAD and fBOS group is calculated with Kruskal-Wallis analysis of variance, or chi-square test when appropriate. Post hoc test is performed with Dunn's multiple comparison test. Significance: [a]$p < 0.05$, [b]$p < 0.01$.

Statistical analysis

Results are expressed as median (interquartile range). Significances among the nCLAD/NRAD, fBOS, and stable groups were tested by Kruskal-Wallis 1-way analysis of variance (ANOVA) in combination with Dunn's multiple comparison post hoc test, and contingency tables were evaluated using the Fisher exact test. Correlation analysis was performed with the Spearman rank test using Prism 4.1 software (GraphPad, San Diego, CA).

Results

Patient characteristics

Patient characteristics are described in Table 1. No significant differences were observed for age, sex, type of LTx, underlying disease pattern, immunosuppressive therapy, the total number of acute rejection episodes, the post-operative day of the BAL sample, and clinical markers of gastro-esophageal reflux (GER). The ischemic time was, however, shorter in CLAD compared with stable patients ($p = 0.03$). Timing of BAL sampling was somewhat (although not significantly) earlier in nCLAD/NRAD than in fBOS patients.

BAL cell count

Cellular differentiation is presented in Table 2. Total cell number was different between nCLAD/NRAD, fBOS, and stable patients, and this difference could be attributed to an increase in cell numbers in the nCLAD/NRAD group.

CLAD patients demonstrated a decrease in percentages of macrophages ($p = 0.0039$) and an increase in percentage of neutrophils ($p = 0.0033$) and in eosinophils ($p = 0.016$). There were no differences in percentage of lymphocytes. The difference in percentage neutrophils was due to a higher percentage in the nCLAD/NRAD and not in the fBOS

Table 2 Cell and Protein Profile in Bronchoalveolar Lavage

Protein (pg/ml)	Stable ($n = 10$) Median (IQR)	CLAD ($n = 18$) Median (IQR)	nCLAD/NRAD ($n = 9$) Median (IQR)	fBOS ($n = 9$) Median (IQR)	ANOVA	nCLAD vs fBOS p-value
Fibronectin ($\times 10^3$)	31.3 (6.0–132.2)	34.9 (8.5–204.5)	27.4 (7.3–78.0)	71.8 (9.6–249.9)	0.73	NA
GROα	517 (148–955)	668 (240–1016)	422 (44–893)	753 (341–1119)	0.57	NA
PLGF	4.4 (3.2–6.0)	5.0 (3.3–7.5)	5.6 (3.3–11.3)	4.7 (3.3–6.5)	0.54	NA
VEGF	425 (250–491)	346 (248–506)	438 (288–608)	289 (61–498)	0.38	NA
TNF-α	3.1 (1.6–3.6)	2.3 (1.7–3.1)	2.1 (0.9–2.9)	3.0 (2.1–3.6)	0.12	NA
FGFb	46.0 (37.5–55.7)	54.0 (44.3–67.1)	57.9 (50.9–69.6)	53.3 (34.5–58.1)	0.083	NA
TGF-β1	36.8 (8.4–51.4)	1.9 (1.9–43.0)	1.9 (1.9–12.9)	1.9 (1.9–80.6)	0.077	NA
MCP-1	97 (40–206)	128 (53–775)	703 (213–1332)	61 (35–128)	0.012	<0.01
RANTES	0.7 (0.2–8.2)	4.3 (1.2–15.0)	15.0 (4.7–26.5)[b]	1.9 (0.6–5.0)	0.0066	<0.05
MMP-9/TIMP-1	1.2 (0.6–4.2)	4.3 (1.4–14.6)[a]	11.8 (5.5–28.7)[b]	2.3 (1.0–4.3)	0.005	<0.05
MMP-9 ($\times 10^3$)	5.0 (2.4–31.2)	215.4 (8.6–494.5)[b]	491.8 (474.4–524.5)[c]	10.7 (5.3–26.8)	0.0007	<0.01
IL-1β	0.4 (0.2–2.7)	2.0 (0.5–26.8)[a]	21.3 (3.6–41.5)[c]	0.6 (0.3–2.2)	0.0004	<0.01
TIMP-1 ($\times 10^3$)	4.3 (2.4–10.2)	13.6 (4.4–45.6)[a]	41.3 (19.4–130.0)[c]	5.0 (3.0–10.8)	0.0004	<0.01
MMP-8/TIMP-1	0.9 (0.4–2.2)	4.5 (1.7–15.1)[b]	15.1 (8.1–21.7)[b]	1.8 (1.0–4.5)	0.0003	<0.05
IL-8	47 (19–129)	258 (91–966)[b]	954 (681–5295)[c]	110 (41–175)	0.0001	<0.01
HGF ($\times 10^3$)	0.2 (0.1–0.6)	1.3 (0.2–7.6)[a]	7.4 (3.2–11.0)[c]	0.2 (0.1–0.5)	0.0001	<0.001
MMP-8 ($\times 10^3$)	3.8 (1.6–15.6)	123.3 (11.0–691.7)[b]	629.8 (366.3–836.0)[c]	11.5 (7.3–14.5)	<0.0001	<0.01
MPO ($\times 10^3$)	1.2 (0.5–4.0)	12.6 (3.4–25.2)[c]	24.6 (17.9–26.5)[c]	3.8 (1.6–7.0)	<0.0001	<0.01
Bile acids (μmol/liter)	0.0 (0.0–0.0)	0.0 (0.0–0.29)	0.13 (0.0–0.84)[a]	0.0 (0.0–0.0)	0.010	<0.05
SP-C	113 (55–293)	32 (32–119)[a]	32 (32–32)[b]	118 (32–152)	0.011	>0.05
RAGE	1497 (764–3459)	199 (31–1590)[a]	48 (20–815)[b]	952 (184–2226)	0.0099	>0.05
PDGF-AA	7.1 (5.1–8.2)	2.1 (0.5–7.5)[a]	0.8 (0.5–1.6)[b]	6.3 (1.3–10.7)	0.0058	<0.05
Total cells (10^3/ml)	50 (30–161)	79 (49–821)	650 (100–1780)[a]	67 (27–79)	0.017	<0.05
% Neutrophils	1.2 (0.4–4.4)	37.4 (3.4–60.0)[b]	57.6 (45.2–76.4)[c]	3.5 (1.9–5.4)	0.0001	<0.01
% Macrophages	94 (85–97)	69 (37–83)[b]	38 (18–50)[c]	83 (81–95)	0.0002	<0.01
% Lymphocytes	3.4 (2.6–11.0)	2.9 (1.4–9.9)	2.4 (1.3–4.5)	8.4 (2.7–11.8)	0.13	NA
% Eosinophils	0.0 (0.0–0.0)	0.3 (0.0–1.1)[a]	0.2 (0.1–0.9)	0.4 (0.0–1.2)	0.033	>0.05

CLAD, chronic lung allograft dysfunction; fBOS, fibrotic bronchiolitis obliterans syndrome; nCLAD, neutrophilic CLAD; NRAD, neutrophilic reversible allograft dysfunction. See Abbreviations for other expansions.

The variation between the stable, nCLAD and fBOS group is calculated with Kruskal-Wallis ANOVA and the Dunn's multiple comparison test is used as post-hoc test for significances of the nCLAD and fBOS group vs the stable group. The significance between the stable and the CLAD group is tested with a Mann Whitney U test. Significance [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$.

Table 3 Correlation Analysis Between Bronchoalveolar Lavage Proteins/Bile Acids and Bronchoalveolar Lavage Neutrophilia

Protein	MCP-1	RANTES	IL-1β	IL-8	TIMP-1	MMP-8	MMP-9	FGFb
P	0.0011	0.023	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0014
R	0.59	0.47	0.82	0.86	0.98	0.90	0.80	0.58
	PLGF	HGF	TGF-β1	MPO	RAGE	SP-C	Bile acids	
P	0.019	<0.0001	0.056	<0.0001	0.023	0.0081	0.0059	
R	0.45	0.90	−0.37	0.82	−0.43	−0.49	0.51	

All significant correlations are displayed with their respective *p*-value and Spearman coefficient. See Abbreviations for expansions.

group. The difference in percentage of macrophages was due to a lower percentage in the nCLAD/NRAD group.

Protein expression in BAL

There were no significant differences detectable in the protein levels of TNF-α, GROα, fibronectin, PLGF, VEGF, FGFb and TGF-β1, although the latter 2 tended to be different (Table 2). The concentrations of IL-8 ($p = 0.0032$), IL-1β ($p = 0.027$), HGF ($p = 0.033$), MMP-8 ($p = 0.0020$), MMP-9 ($p = 0.020$), MMP-8/TIMP-1 ($p = 0.0048$), MMP-9/TIMP-1 (0.026), and MPO ($p = 0.0012$) were higher in the CLAD group than in the stable group, whereas the levels of RAGE ($p = 0.029$), SP-C ($p = 0.041$), and PDGF-AA ($p = 0.049$) were lower in the CLAD group. These differences were all caused by a different protein concentration in the nCLAD/NRAD group compared with the stable group; there were no differences between the fBOS and the stable groups.

There were significant differences in IL-1β, IL-8, HGF, MCP-1, RANTES, MMP-8, MMP-9, TIMP-1, MPO, TIMP-1, and PDGF-AA in the nCLAD/NRAD group compared with the fBOS group. See Table 2 for more detailed information and Figure 3 for representations of relevant proteins.

Bile acids in BAL

Although there was no difference in pH-impedance and GER parameters between groups, bile acid levels were only elevated in the nCLAD/NRAD group compared with the fBOS group (Table 2). There was no difference between the CLAD and the stable group.

Correlation analysis

MCP-1, RANTES, IL-1β, IL-8, TIMP-1, MMP-8, MMP-9, FGFb, PLGF, HGF, MPO, RAGE, SP-C, and bile acids were all significantly correlated with the percentage of neutrophils in BAL (Table 3).

Discussion

Our results corroborate previously published data and demonstrate that inflammation (IL-8, IL-1β, MCP-1, RANTES), matrix remodelling (TIMP-1, MMP-8, MMP-9), growth factors (HGF, PDGF-AA), oxidative stress (MPO, SP-C), and epithelial damage (RAGE) are involved in patients with CLAD. Here, we provide evidence that these proteins are differentially expressed in BAL fluid of the nCLAD/NRAD group but not in fBOS. On top of this, bile acids were exclusively increased in nCLAD/NRAD.

In this study we measured a wide range of proteins reflecting different processes in patients with CLAD after LTx. This study also corroborates earlier studies of the effect of azithromycin in nCLAD/NRAD patients who experienced a mean increase in FEV_1 of 21% after 3 months of treatment with azithromycin ($p = 0.0039$), all returning to a normal pulmonary function. Although the mean BAL neutrophilia in these NRAD patients was high (58%), there was no evidence of an active bacterial, fungal, or viral infection at the time of BAL.

There were no differences in patient characteristics and immunosuppressive therapy except for the ischemia time between groups, explained by the somewhat higher number of single LTxs in the nCLAD/NRAD group. There was also a tendency for an earlier onset in FEV_1 decline in nCLAD/

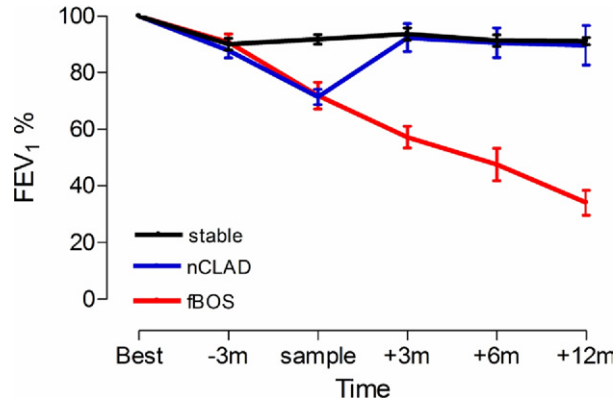

Figure 2 The forced expiratory volume in 1second (FEV_1) evolution in the 10 stable, 9 nCLAD, and 9 fBOS patients. Spirometry data of all patients are available at each interval. The nCLAD patients experienced a significant ($p = 0.0039$) mean increase in FEV_1 of 21% after 3 months of treatment. During the same period, the FEV_1 of fBOS patients further deteriorated by 15% ($p = 0.014$). The stable patients remained stable during at least 1 year after the BAL sample. CLAD, chronic lung allograft dysfunction; fBOS, fibrotic bronchiolitis obliterans syndrome; nCLAD, neutrophilic CLAD; NRAD, neutrophilic reversible allograft dysfunction.

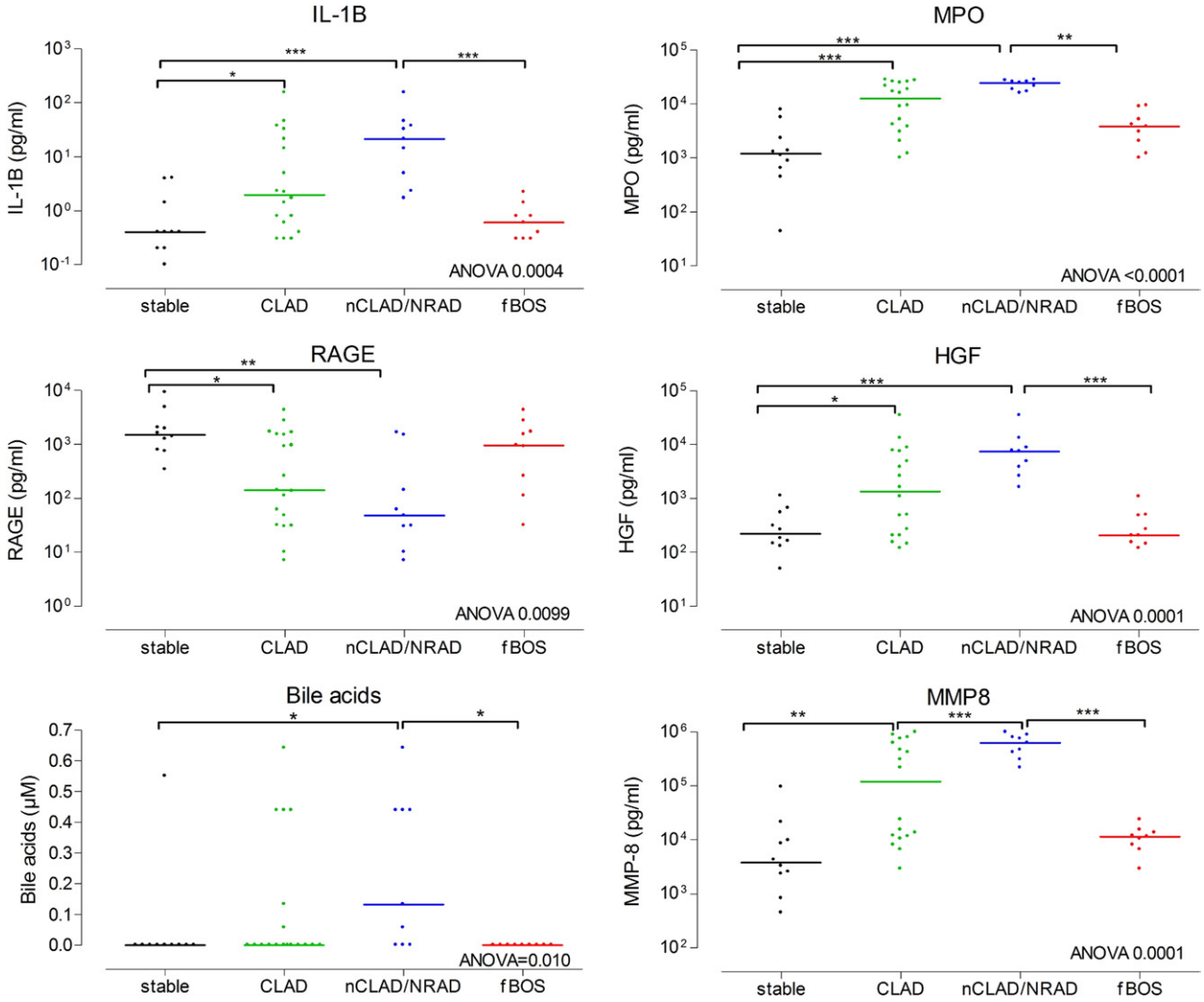

Figure 3 The expression of (A) IL-1β, (B) MPO, (C) MMP-8, (D) RAGE, (E) HGF (see Abbreviations table) and (F) bile acids in BAL fluid in the 10 stable, 18 CLAD, 9 nCLAD/NRAD, and 9 fBOS patients. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CLAD, chronic lung allograft dysfunction; fBOS, fibrotic bronchiolitis obliterans syndrome; nCLAD, neutrophilic CLAD; NRAD, neutrophilic reversible allograft dysfunction.

NRAD compared with fBOS patients. Indeed, it is accepted that persistent BAL neutrophilia, occurring early after LTx, is a risk factor for BOS (now called CLAD).[7,10,11] As a consequence, nCLAD/NRAD may be seen as a risk factor for BOS, although by itself it can no longer be classified as BOS, according to current definition. Moreover, about 30% of patients who developed nCLAD/NRAD and improved their FEV_1 with azithromycin may subsequently develop fBOS.[3,12]

Because nCLAD/NRAD is characterized by a high percentage of neutrophils in BAL, it seems obvious that typical neutrophilic inflammation and matrix remodelling factors are differentially regulated. It is, however, important to remark that none of these proteins is significantly different in the BAL of fBOS compared with stable patients. This is also reflected in the correlation analysis that showed a sometimes-strong association with the percentage of neutrophils in BAL. This demonstrates that all previous data regarding neutrophilia and cytokine and chemokine upregulation in BOS/CLAD need to be interpreted with great caution, because most studies performed up to now have not differentiated between nCLAD/NRAD and fBOS. This lack of differentiation leads to biased results because most positive results are possibly due to the nCLAD/NRAD group. Indeed, an upregulation in gelatinase activity in BOS patients was recently described.[13] After re-evaluation of their data in response to a question from our group,[14] the authors acknowledge that this difference was due to patients with high BAL neutrophilia levels within their BOS group, whereas the low BAL neutrophilia group did not show any difference compared with the stable group.[15]

Bile acids were only increased in nCLAD/NRAD patients and not in fBOS and stable patients, whereas evidence of GER was not different between the groups. This is in agreement with findings of D'Ovidio et al,[16] who observed an elevation in bile acids in early BOS, characterized by increased BAL neutrophilia, compared with late BOS. Moreover, there was a correlation between BAL neutrophilia and bile acids,[16] which we confirmed in the present study. This could indicate that

non-acidic microaspiration is a possible trigger for the accumulation of neutrophils in BAL.

Intriguing is the downregulation of SP-C in nCLAD/NRAD. Surfactant proteins are involved in maintaining surface tension within the lungs and in protecting against inflammation and injury in BOS. High bile acid levels are associated with low SP-A and SP-D levels and the development of BOS.[17] Our results corroborate that the combination of a downregulation in surfactant protein C and an elevation of bile acids is important in nCLAD/NRAD but not in fBOS.

Changes in RAGE, PDGF-AA, and TGF-β1 are difficult to interpret because they are generally thought to be pro-inflammatory,[18] although anti-inflammatory effects are also described.[19] There are even reports that downregulation of RAGE could trigger the transition from chronic inflammation to fibrosis,[20] which seems to be the case in this nCLAD/NRAD phenotype because it often proceeds the development of BOS.

It would have been very interesting to evaluate the effect of azithromycin treatment on all differentially regulated BAL proteins in the nCLAD/NRAD group, but we do not have follow-up samples after 3 to 6 months treatment for all of our patients. We previously demonstrated that BAL neutrophilia and IL-8 levels in nCLAD/NRAD patients decreased after treatment,[5] and we speculate that other reported proteins in this study may behave similarly, although this needs further investigation. At least, we did find a significant decrease in MMP-9 levels in BAL of nCLAD/NRAD patients after 3 months of treatment with azithromycin (unpublished results).

This study, however, has some limitations because only a limited number of samples were used, and more importantly, BAL sampling may not be the ideal tool to study fBOS. Other techniques, such as immunohistochemistry on biopsy samples or blood analysis, could help us to further unravel mechanisms involved in fBOS.

In conclusion, our results clearly indicate that the BAL protein levels and hence the pathophysiology of nCLAD/NRAD and fBOS seems completely different. Indeed nCLAD/NRAD should no longer be classified as BOS due to the reversible nature of the disease. Most of the proteins that were upregulated in BOS patients according to existing literature, in fact, seem to be explained by nCLAD/NRAD. In patients with fBOS, the mechanisms remain unclear (at least when analyzing BAL protein levels with the current techniques) and remain to be further investigated. (Figures 2–3).

Disclosure statement

Dr G.M. Verleden is holder of the Glaxo Smith Kline (Belgium) chair in respiratory pharmacology at the Katholieke Universiteit (K.U.) Leuven and is supported by the Research Foundation Flanders (FWO): G.0643.08 and G.0723.10 and 'Onderzoeksfonds K.U. Leuven (OT/10/050). Dr Vanaudenaerde, Dr Van Raemdonck, and Dr Dupont are senior research fellows of the FWO.

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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