Profiling the dichotomy of Bronchiolitis Obliterans Syndrome after lung transplantation

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At a glance commentary: - scientific knowledge on the subject: chronic rejection remains a major complication after lung transplantation. We recently demonstrated the existence of a dichotomy within chronic rejection with an inflammation phenoytype and a fibrotic phenotype.

- What this study adds to the field: These data confirm that NRAD and fBOS should be regarded as 2 totally different entities with different pathophysiological mechanisms.

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ABSTRACT

Rationale: Bronchiolitis Obliterans Syndrome (BOS) remains a major cause of mortality after lung transplantation. Previous data suggested the existence of at least 2 phenotypes (NRAD, Neutrophilic reversible allograft dysfunction and fBOS, fibroproliferative BOS) within BOS.
Objectives: We aim to further unravel the pathophysiological mechanisms involved in these phenotypes by measuring protein expression of different markers in broncho alveolar lavage (BAL).
Methods: BAL samples of 29 lung recipients were included in this study. Patients were subdivided based on the absence/presence of BOS and subsequently by their response to azithromycin resulting in 3 different groups: stable (n=10), fBOS (n=10) and NRAD (n=9). 32 different proteins were measured by single and multiplex ELISA.
Measurements and main results: Our results show that inflammation (IL-8), 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 BOS. We provided evidence that these variations are predominantly caused by the NRAD group while in the fBOS group only MPO was upregulated. MCP-1, RANTES, IL-1β, IL-8, TIMP-1; MMP-8, MMP-9, HGF, MPO, and bile acid concentrations are upregulated in NRAD compared to fBOS whereas RAGE, SP-C and PDGF-AA are downregulated in NRAD compared to fBOS.
Conclusion: These data confirm that NRAD and fBOS should be regarded as 2 separate entities with different pathophysiological mechanisms, which may need different therapeutical approaches.

Keywords: Bronchiolitis Obliterans Syndrome, fibroproliferative BOS, lung transplantation, markers, mechanisms, neutrophilic reversible allograft dysfunction, risk factors

Abstract word count 222 (250)

INTRODUCTION

Lung transplantation is the ultimate treatment for patients with end-stage lung diseases like chronic obstructive pulmonary disease, interstitial pulmonary fibrosis, pulmonary arterial hypertension and cystic fibrosis. The survival, however, is hampered by the manifestation of Bronchiolitis Obliterans Syndrome (BOS). BOS is defined as an irreversible decline in forced expiratory volume in 1 second (FEV_1) by at least 20% in the absence of other identifiable causes (1). It affects 45-50% of the lung transplant (LTx) patients after 5 years and accounts for 30% of the late mortality (2). The mechanism of BOS is commonly accepted to be both an alloantigen-dependent and an alloantigen-independent injury of the epithelium leading to an influx of inflammatory cells, inducing tissue damage and finally fibrosis (3, 4). Increased luminal neutrophilia or the presence of markers that reflect neutrophil infiltration in the airways, are believed to be characteristic for BOS patients (3). Upon activation, neutrophils release numerous mediators such as myeloperoxidase (MPO) and metalloproteinases (5, 6).

The neomacrolide azithromycin was the first drug that was able to improve the lung function in about 40% of BOS patients (7). Based on the response to azithromycin, our group identified the existence of at least 2 different phenotypes within BOS (8, 9). The first phenotype, characterized by airway neutrophilia, FEV_1 improvement $\geq$10% after azithromycin treatment, an early onset and a gradual FEV_1 decline is termed Neutrophilic Reversible Allograft Dysfunction (NRAD), the other phenotype demonstrated no airway neutrophilia, no FEV_1 improvement after azithromycin thereapy, a rather late onset (after 2 years) and a rapid FEV_1 decline. This phenotype was termed fibroproliferative BOS (fBOS) and is thought to reflect more the pathological correlate, obliterative bronchiolitis (10).

The clinical presentation of these phenotypes was previously reported but there is no real insight in the different pathophysiological mechanisms. Although it is accepted that BOS in general in characterised by increased airway neutrophilia and IL-8 levels in BAL, we clearly demonstrated that this was not the case in the fBOS phenotype, but only in NRAD. As a consequence we aim to further profile the mechanisms in both phenotypes by looking at different proteins reflecting a range of processes previously reported to be involved in BOS such as inflammation, oxidative stress, angiogenesis, matrix remodeling, reflux and fibrosis. Improved understanding of the disease mechanisms could provide us with valuable information especially for potential treatment of BOS and could pave the way for a better long term survival after lung transplantation.

Introduction 403 (450)

MATERIALS AND METHODS

Patient characteristics

Since October 2001, all LTx recipients in our centre are enrolled in a routine prospective bronchoscopy study. Physical examination, radiological examination, FEV_1, and bronchoscopy with bronchoalveolar lavage (BAL) are performed around fixed time points: 21, 90, 180, 360, 540, 720, 1080, 1800 days after transplantation, or when acute rejection, infection or BOS are suspected.

To create the most clear cut group of NRAD and fBOS patients/samples needed to full-fill several criteria: diagnosis of BOS≥1 according to the ISHLT criteria (11), a bronchoscopy with BAL and transbronchial biopsy in the proximity of this time point of BOS diagnosis and azithromycin therapy started shortly thereafter. The NRAD group typically showed an FEV_1 increase of at least 10-15% after azithromycin therapy, whereas the fBOS group did not respond and experienced a further detoriation in FEV_1 (figure 1). A control group (free of BOS) was selected and matched according to the postoperative time of BAL in the whole BOS group. Patient characteristics are described in table 1. The 3 patient groups were retrospectively selected, in order to have a clearcut phenotypical diagnosis.

Lung function, BAL and bronchoscopy

FEV_1 measurement is performed as previously described and according to American Thoracic Society guidelines (12). For BAL, two aliquots of sterile 50 mL saline were instilled in the right middle lobe or lingula. The returned fractions were pooled and processed as previously described (13). Total and differential cell counts are routinely performed in the lab as previously reported (13). Another fraction was immediately centrifuged at 500g for 10 min at 4° and then stored at -80°C. The supernatant was used for protein measurement. Transbronchial biopsy specimens were always taken after the BAL procedure in right or left lower lobe. Specimens were examined by a pathologist skilled in LTx and graded according to the ISHLT guidelines (14).

Clinical gastro-oesophageal reflux parameters and bile acid measurement

pH impedance measurement was performed as previously described to measure the total number of reflux events, the bolus exposure and the acid exposure (15). When no assessment was available, gastroscopy data was used to determine whether or not the patient was suffering from reflux esophagitis. Bile acids were quantified in 3µl of undiluted BAL fluid in

duplicate with a commercially available enzymatic assay (Bioquant, San Diego, USA). The detection limit was 0.2 µM.

Protein profiling by Search light methodology

Analysis of human protein expression was measured in the supernatant of the BAL fluid. 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; SDF-1) were detected using the custom multiplex SearchLight® Assay System (Aushon, Billerica, MA). Searchlight technology is based on a traditional sandwich ELISA technique which integrates plate-based antibody arrays with chemiluminescent detection. 400 µl of 12 selected samples (4 of each group) were shipped as a pilot experiment on dry ice to the Searchlight Sample Testing Service, who performed the assays (Aushon, Billerica, MA). All samples are analysed in duplicate. IFN-γ, IL-4, IL-10, IL-12p40, IL-17A, IL-23, SDF-1 and cox-2 were under the detection limit. IL-6, osteopontin, GM-CSF, G-SCF and EGF were also left out because in each group too few samples revealed differences.

In the final experiment 400 µl of 29 samples (10 stable, 9 NRAD, 10 fBOS) were shipped to the searchlight testing service for measuring 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.

Statistical analysis

Results are expressed as median (interquartile range) or as mean (± standard error of mean). Significances between groups were tested by using Kruskal-Wallis one way analysis of variance (ANOVA) in combination with Mann Whitney U *post hoc* test. Contingency tables were evaluated using the Fisher's Exact test. Statistics was performed using Prism 4.1 software (San Diego, CA, USA)

Methods 642 (500)

RESULTS

Patient characteristics

Patient characteristics are described in table 1. No differences were observed for age, sex, type of Tx, underlying disease pattern, immunosuppressive therapy, the number of episodes of acute rejection prior to BOS onset, chronic colonization with Pseudomonas or Aspergillus in BAL, the rate of infections prior to BOS onset, the post operative day of the BAL sample and clinical markers of gastro-oesophageal reflux (GER). The ischemic time was significantly different between BOS and stable patients (p=0.03). The ischemic time in NRAD patients was lower compared to the stable group (p=0.0021) but not versus fBOS (p=0.053).

BAL cell count

Cell profiles are presented in table 2. BOS patients demonstrated a decrease in % macrophages (p=0.003), an increase in % neutrophils (p=0.0058) and % eosinophils (p=0.011). There were no differences in % lymphocytes. The difference in % macrophages is completely due to the NRAD group (p<0.0001). The % neutrophils and % eosinophils are different in both the NRAD (p<0.0001 and p=0.017) and fBOS (p<0.0001 and p=0.04) group compared to the stable group with no differences between the groups (figure 1B).

Total cell number was different between NRAD, fBOS and stable patients (p=0.019) and this difference can be attributed to an increase in cell numbers in the NRAD group (p=0.017).

Inflammation parameters in BAL

Higher levels of IL-8 (p=0.002) and IL-1β (0.025), but not MCP-1, RANTES, TNF-α and GROα were observed in the BOS group versus the stable group (table 2).

The NRAD group showed higher levels of MCP-1 (p=0.013 and p=0.016), RANTES (p=0.0057 and p=0.001), IL-1β (p=0.0005 and p<0.0001) and IL-8 (p<0.0001 and p<0.0001) compared to the stable and the fBOS group respectively. There were no differences between the fBOS and the stable group.

Matrix remodelling parameters in BAL

Higher levels of MMP-8 (p=0.0014), MMP-9 (p=0.0096), TIMP-1 (p=0.012), MMP-8/TIMP1 (0.0046) and MMP-9/TIMP1 (p=0.021) were observed in the BOS group compared to the stable group (table 2).

The NRAD group is marked with higher levels of MMP-8 (p<0.0001 and p<0.0001), MMP-9 (p=0.0021 and p=0.0015), TIMP-1 (p=0.0015 and p=0.0004), MMP-8/TIMP-1 (p=0.0002 and

0.0011) and MMP-9/TIMP1 (p=0.0041 and p=0.02) compared to the stable and the fBOS group respectively. No differences were present between the fBOS and the stable group.

Oxidative stress in BAL

MPO was higher in the BOS group compared with the stable group (p=0.0009) (table 2).

Both NRAD and fBOS group showed higher concentrations of MPO (p<0.0001 and p=0.035) compared to the stable group. The MPO levels of the NRAD group, however, was higher than the fBOS group (p<0.0001).

Growth factors in BAL

An increase in HGF (p=0.023), a decrease in PDGF-AA (p=0.037), a tendency towards a decrease in TGF-β (p=0.06) but not for FGFb, VEGF and PlGF was observed in the BOS group versus to the stable (table 2).

The NRAD group revealed higher levels of HGF and lower levels of PDGF-AA compared to the stable (p<0.0001 and p=0.0002) and fBOS (p<0.0001 and p=0.034) groups. No differences between the fBOS and the stable group were present.

RAGE, SP-C and fibronectin in BAL

RAGE (p=0.021) and SP-C (p=0.033), but not fibronectin, were lower in the BOS group versus the stable group (table 2).

RAGE was downregulated in the NRAD group compared to the stable (p=0.041) and fBOS group (p=0.028). SP-C levels in the NRAD group were lower compared to the stable group (p=0.003), but not compared to the fBOS group (p=0.089). No differences between the fBOS and the stable group were present.

Bile acids in BAL

Bile acids were not different in the BOS group versus the stable group (table 2).

Bile acids levels were however higher in the NRAD group compared to the fBOS group (p=0.017). There was no difference between the NRAD and fBOS group compared to the stable group.

DISCUSSION

Our results show that inflammation (IL-8), 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 BOS. We provided evidence that these variations are predominantly caused by the NRAD group, while in the fBOS group only MPO was upregulated. MCP-1, RANTES, IL-1β, IL-8, TIMP-1; MMP-8, MMP-9, HGF, MPO, and bile acid concentrations are upregulated in NRAD compared to fBOS, whereas RAGE, SP-C and PDGF-AA are downregulated in NRAD compared to fBOS. These results consolidate the existence of a dichotomy within BOS with 2 mechanistically different phenotypes.

Our patient group proved to be well-chosen as demonstrated by figure 1. Indeed, in retrospect the stable patients remain at their maximum FEV_1 for at least one year after sampling. fBOS patients further detoriated: one patients died of established obliterative bronchiolitis, a second one was retransplanted and 2 others are currently on the waiting list for retransplantation. All NRAD patients had a mean increase in their FEV_1 of 27% after 3 to 6 months of treatment with azithromycin. After 1 year, this FEV_1-increase was still 20% compared to sampling time. Besides their response in FEV_1, the percentage neutrophils and the total cell count in BAL before azithromycin therapy were also higher in the NRAD group compared to the fBOS group. We matched the median timing of the BAL sampling for the stable and the BOS group, but still there was a trend towards a later onset of the FEV_1 decline in the fBOS group compared to the NRAD group. These data are all in agreement with previous data from our group (10). There were no differences in patient characteristics and immunosuppressive therapy except for the ischemia time, explained by the somewhat higher number of single lung transplantations in the NRAD group. Remarkable however is the fact that potential risk factors as colonisation or frequent acute rejection, related to the development of BOS, are not significant in this study. This is probably due to the inclusion of only 29 patients in this study.

A large number of studies have already demonstrated an important link between BAL neutrophilia, its prime chemo-attractant IL-8 and BOS (16,17,18). Our group further refined this as only a subset of the BOS patients (NRAD) showed an increased BAL neutrophilia and IL-8, while the fBOS group showed levels comparable to stable patients (8). This study confirmed the involvement of many other proteins in BAL. Subdividing the BOS group into NRAD and fBOS clearly attributed the differences to the NRAD group, while the fBOS group showed no difference at all. IL-8 is not the only chemo-attractant associated with BOS, MCP-1 and RANTES for example are reported to be involved (19) as is also confirmed in our results. Their main aim is to attract monocytes and to a lesser extent dendritic cells and

memory T-cells. Dendritic cells are able to differentiate naive T cells into IL-17 producing T_H17 cells. IL-17 is thought to be important in BOS (13) and we recently demonstrated IL-17 positive cells in the submucosa of NRAD patients (20), which are likely T_H17 cells. To differentiate into T_H17 cells, IL-1β and IL-6 are required in humans, while TGF- β seems to have an inhibitory effect (21). Our results point towards a T_H17 promoting environment (upregulation of IL-1β, downregulation of TGF-β1 although not significant) in the NRAD group. One may therefore assume that TH17 cells are the main inducers of this neutrophilic inflammation in NRAD. Neutrophils are important as they release numerous factors, among which MMP-8 and MMP-9. Matrix remodelling has a long history of association with BOS even leading to a possible use of MMP-9 as a marker for BOS in BAL (22). The difference between stable and BOS patients however is almost exclusively caused by NRAD patients. This is caused by the high abundance of neutrophils. MMPs together with their inhibitors (TIMP) are crucial for turnover of the extracellular matrix and it seems that matrix remodelling is one of the key players during inflammation- type BOS but not in the fibrotic type.

Although inflammation and angiogenesis are thought to coincide, the markers for angiogenesis were not increased in BOS. The concentration of VEGF and FGF was not different in the BOS group and PDGF was even downregulated, which is not in line with current literature. Langenbach *et al.* stated that in all lung transplant recipients (even the stable one) airway vascular changes occur at an early stage (23). There is, however, an increase in airway vessel number in BOS, although not reflected by higher concentration of typical angiogenesis markers in BAL. On top of this anti-VEGF antibodies could not decrease angiogenic activity. The authors suggested that IL-8, which is upregulated, could be the driving force for angiogenesis in BOS and not VEGF (24).

Aharinejad *et al.* demonstrated an upregulation of HGF, a key player in regeneration of injured lung tissue, in serum of patients with acute rejection compared to stable patients (25). Recently these data were confirmed in BAL as there is an elevation of HGF levels in BOS patients (26). This could reflect a reparative response to the recurrent injury of the epithelia. In our study, the HGF levels in BAL were indeed elevated in BOS patients, but again merely in the NRAD patients.

Next to angiogenesis and inflammation, oxidative stress is involved early in the development of BOS (27) as reproduced by our results as increased MPO levels both in NRAD and fBOS are seen, although much more pronounced in NRAD. Macrolides have the ability to decrease oxidative stress, generated by neutrophils and structural cells like airway smooth muscle cells

(28), next to their known role in decreasing neutrophils. As a consequence part of the beneficial effect of azithromycin may be due to its anti-oxidative effect.

Surfactant proteins are involved in maintenance of the surface tension within the lungs and protection against inflammation in BOS. D' Ovidio *et al.* demonstrated a link between high bile acid levels and low SP-A and D levels and freedom from BOS (29). Bile acids have a negative dose dependent effect on the surfactant proteins. It seems however from our results that this relationship is again only valid for the NRAD patients.

PDGF RAGE TGF....

Although this study adds further evidence for the existence of a dichotomy within BOS, it has some drawbacks. Firstly, this study is performed with 29 samples only. Since the difference between the BOS phenotypes are that clear, we do not suspect that higher numbers per group would completely reverse the results. Secondly, not all proteins are measurable in BAL fluid and therefore the use of biopsy samples could prove to be more effective in demonstrating the dichotomy. The problem however is that biopsy sampling in OB/BOS patients has a very low sensitivity.

In conclusion, our results indicate that the definition of BOS is currently insufficient. The mechanisms of action of the different phenotypes seem to be so different that they cannot be pooled under one term BOS. There is a clear need for a revision and a re-evaluation of the definition of BOS. Most of the proteins that were upregulated in BOS patients according to the present literature, in fact seem to be due to the NRAD phenotype in this study. In patients with the fBOS phenotype, which is more likely to reflect the real OB, the mechanisms remain unclear and unsolved and effective therapy is unfortunately still lacking.

Figure Legends

Figure I: The FEV_1 evolution (A) and percentage of neutrophils in the BAL (B) in the stable (n=10), BOS (n=19), NRAD (n=9) and fBOS (n=10) group. FEV_1 increases in the NRAD group with administration of azithromycin at diagnosis of BOS while the fBOS group further declines. BAL neutrophilia at the onset of BOS is higher in the NRAD group compared to the fBOS and stable group.

Figure II: The expression of IL-1β, MMP-9, MPO, RAGE, HGF and bile acids in BAL fluid in the stable (n=10), BOS (n=19), NRAD (n=9) and fBOS (n=10) group.

TABLES AND FIGURES

Figure I

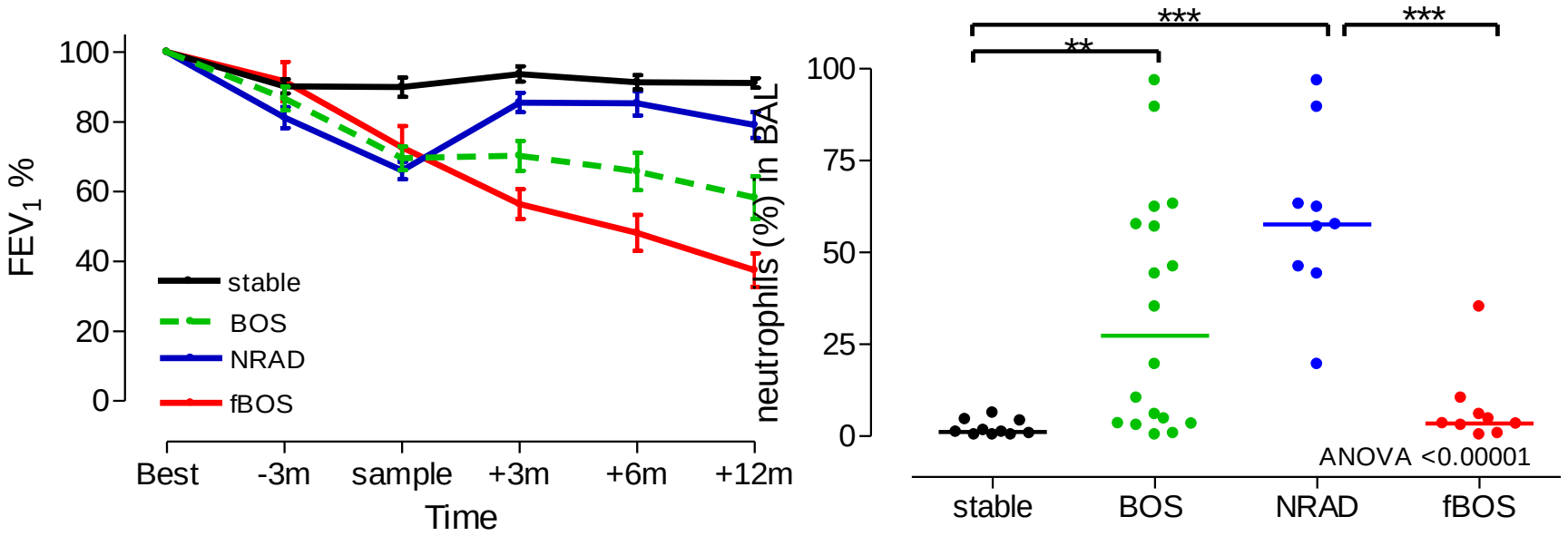

Figure II

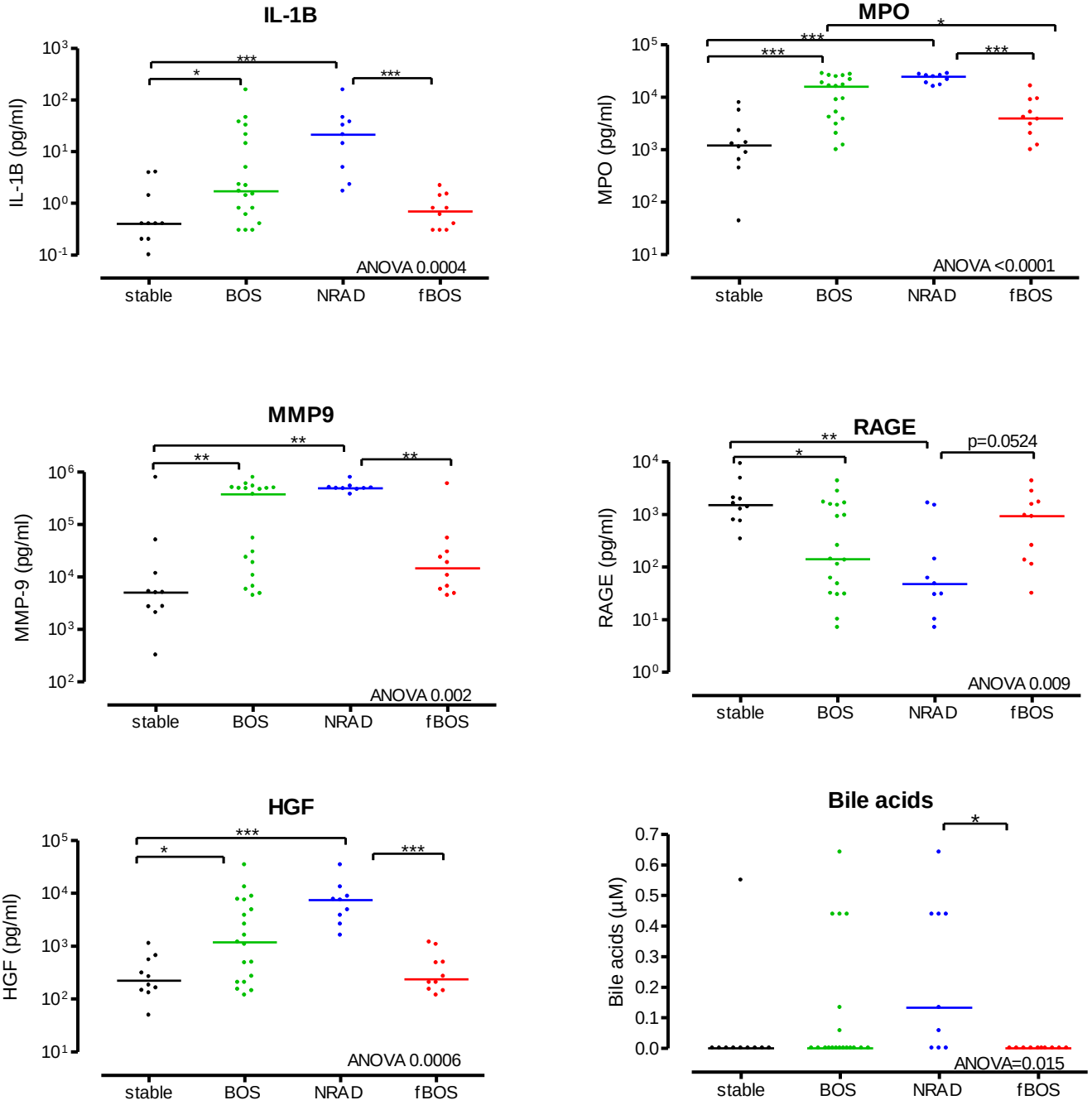
IL-1B
IL-1B (pg/ml)
ANOVA 0.0004
stable
BOS
NRAD
fBOS
MPO
MPO (pg/ml)
ANOVA <0.0001
MMP9
MMP-9 (pg/ml)
ANOVA 0.002
RAGE
RAGE (pg/ml)
p=0.0524
ANOVA 0.009
HGF
HGF (pg/ml)
ANOVA 0.0006
Bile acids
Bile acids (µM)
ANOVA=0.015

Table I: Patient characteristics

	Stable (n=10)	BOS (n=19)		NRAD (n=9)	fBOS (n=10)	p-value
Age at TX, years	43 (32-54)	51 (33-57)		52 (49-59)	36 (29-55)	0.11
Sex (Male/Female), n	6/4	9/10		4/5	5/5	0.78
Type of Tx (SSLTX/SLTX/HLTx),n	9/0/1	14/4/1		6/3/0	8/1/1	0.31
Ischemia, hours	6.7 (6.1-7.3)	5.5 (4.6-6.5)*		5.1 (4.3-6.0)**	6.4 (4.9-7.6)	0.02
CMV match/mismatch	4/6	13/6		5/4	8/2	0.19
Pre LTx diagnosis, n						0.34
Emphysema (COPD/α1-ATD)	4/0	7/2		6/1	1/1	
Fibrosis/sarcoidosis	2/1	5/0		1/0	4/0	
PH (primary/Eisenmenger)	1/0	0/1		0/0	0/1	
Cystic fibrosis	2	4		1	3	
Immunosuppresive therapy,n						
Steroids/none	10/0	18/1		8/1	10/0	0.68
FK/CSA	9/1	15/4		8/1	7/3	0.78
AZA/MMF/none	8/0/2	17/2/0		8/1/0	9/1/0	0.55
TBB, n						
Grade A total	0.7±0.2	0.7±0.2		0.9 ± 0.4	0.6±0.2	0.97
Grade A≥2	0.4±0.2	0.2±0.1		0.1 ± 0.1	0.2±0.1	0.33
Grade B total	0.3±0.1	0.2±0.1		0.4 ± 0.2	0.2±0.1	0.87
Grade B≥2	0.2±0.1	0.2±0.1		0.1 ± 0.1	0.2±0.1	0.85
GERD, n						0.69
No assessment	0	3		1	2	
Gastroscopy	1	2		1	1	
pH-Impedance measurement	9	14		7	7	
GER on gastroscopy, n	0	0		0	0	
GER on pH-impedance, n						
Total number of events	47.9±8.5	51.5±9.5		33.9±8.1	69.1±14.8	0.10
Bolusexposure (<1.4%)	1.4±0.4	2.1±0.6		1.4±0.9	2.6±0.8	0.37
Acid exposure (<4.2%)	5.8±3.4	5.7±2.8		6.8±5.5	4.7±1.7	0.94
Colonisation (Ps/Asp/none),n	0/1/9	4/2/13		2/1/6	2/1/7	0.64
Time of BAL, months	17.9±0.1	25.8±3.9		20.0±5.9	31.0±5.0	0.09

Results are expresses as median (IQR) or as mean (±SEM) when appropriate.

NRAD= neutrophilic reversible allograft dysfunction; BOS= Bronchiolitis Obliterans Syndrome; fBOS= fibroproliferative BOS, Tx= transplantation; SSLTX= double lung transplantation, SLTx= single lung transplantation, HLTx= heart- lung transplantation; CMV= cytomegalovirus; COPD= chronic obstructive pulmonary disease; α1ATD= α1 antitrypsin deficiency; PH= pulmonary hypertension; FK= tacrolimus; CSA= cyclosporine; AZA= azathioprine; MMF= mycofenolate mofetil; TBB= transbronchial biopsy, GER= gastro-oesophageal reflux; Ps= pseudomonas; Asp= aspergillus; BAL= bronchoalveolar lavage.

The variation between the stable, NRAD and fBOS group is calculated with Kruskall Wallis ANOVA or with one way- ANOVA or chi-square test when appropriate. Post hoc test is performed with Mann- Whitney U test. Significance *$p<0.05$

protein (pg/ml)	Stable (n=10)	BOS (n=19)	BOS vs stable	NRAD (n=9)	fBOS (n=10)	ANOVA	NRAD vs stable	NRAD vs fBOS	fBOS vs stable
TNF-α	3.1 (1.6-3.6)	2.2 (1.5-3)	0,22	2.1 (0.9-2.9)	2.7 (2.0-3.6)	0.16	NA	NA	NA
MCP-1	97 (40-206)	96 (46-703)	0,13	703 (213-1332)	60 (27-128)	**0.0081**	**0,013**	**0,0016**	0,25
RANTES	0.7 (0.2-8.2)	3.8 (0.9-13.1)	0,07	15.0 (4.7-26.5)	1.5 (0.4-3.8)	**0.0054**	**0,0057**	**0,001**	0,83
IL-1β	0.4 (0.2-2.7)	1.7 (0.6-21.3)	**0,025**	21.3 (3.6-41.5)	0.7 (0.3-1.5)	**0.0003**	**0,0005**	**<0,0001**	0,53
GROα	517 (148-955)	585 (229-948)	0,73	422 (44-893)	669 (253-1119)	0.71	NA	NA	NA
IL-8	47 (19-129)	313 (110-954)	**0,002**	954 (681-5295)	135 (41-193)	**<0.0001**	**<0,0001**	**<0,0001**	0,078
TIMP-1 (*10^3)	4.3 (2.4-10.2)	13.8 (5.0-41.3)	**0,012**	41.3 (19.4-130.0)	6.5 (3.0-15.4)	**0.0004**	**0,0015**	**0,0004**	0,35
MMP-8 (*10^3)	3.8 (1.6-15.6)	20.6 (11.5-629.8)	**0,0014**	629.8 (366.3-836.0)	11.7 (7.3-19.5)	**<0.0001**	**<0,0001**	**<0,0001**	0,063
MMP-9 (*10^3)	5.0 (2.4-31.2)	376.2 (10.7-497.2)	**0,0096**	491.8 (474.4-524.5)	14.7 (5.3-42.4)	**0.0015**	**0,0021**	**0,0015**	0,12
MMP-8/Timp-1	0.9 (0.4-2.2)	4.5 (1.8-15.1)	**0,0046**	15.1 (8.1-21.7)	1.9 (1.3-4.4)	**0.0003**	**0,0002**	**0,0011**	0,11
MMP-9/Timp-1	1.2 (0.6-4.2)	4.5 (1.6-17.3)	**0,021**	11.8 (5.5-28.7)	2.8 (1.1-6.7)	**0.008**	**0,0041**	**0,02**	0,22
FGFb	46.0 (37.5-55.7)	53.7 (46.8-67.0)	0,15	57.9 (50.9-69.6)	52.7 (34.5-58.1)	0.072	NA	NA	NA
PLGF	4.4 (3.2-6.0)	4.7 (4.0-7.4)	0,28	5.6 (3.3-11.3)	4.7 (3.2-6.5)	0.54	NA	NA	NA
VEGF	425 (250-491)	303 (224-499)	0,57	438 (288-608)	280 (61-498)	0.24	NA	NA	NA
HGF (*10^3)	0.2 (0.1-0.6)	1.2 (0.2-7.4)	**0,023**	7.4 (3.2-11.0)	0.2 (0.1-0.8)	**0.0001**	**<0,0001**	**<0,0001**	0,74
TGF-β1	36.8 (8.4-51.4)	1.9 (1.9-33.2)	0,06	1.9 (1.9-12.9)	1.9 (1.9-80.6)	0.074	NA	NA	NA
PDGF-AA	7.1 (5.1-8.2)	2.0 (0.5-7.5)	**0,037**	0.8 (0.5-1.6)	4.4 (1.2-10.7)	**0.0055**	**0,0002**	**0,034**	0,68
Fibronectin (*10^3)	31.3 (6.0-132.2)	32.5 (9.0-166.2)	0,91	27.4 (7.3-78.0)	54.5 (9.6-249.9)	0.73	NA	NA	NA
MPO (*10^3)	1.2 (0.5-4.0)	15.9 (3.8-24.6)	**0,0009**	24.6 (17.9-26.5)	4.0 (1.6-9.1)	**<0.0001**	**<0,0001**	**<0,0001**	**0,035**
RAGE	1497 (764-3459)	141 (32-1535)	**0,0205**	48 (20-815)	931 (124-2226)	**0.009**	**0,0041**	**0,028**	0,22
SP-C	113 (55-293)	32 (32-118)	**0,033**	32 (32-32)	75 (32-1528)	**0.0062**	**0,003**	0,089	0,43
Bile acids (µM)	0.05±0.05	0.11±0.05	0,39	0. 24±0.08	0.00±0.00	**0.015**	0,054	**0,0172**	0,97
Total cells (10^3/ml)	50 (30-161)	81 (53-650)	0,1	650 (100-1780)	70 (30-114)	**0.019**	**0,017**	**0,01**	0,63
% neutrophils	1.2 (0.4-4.4)	37.4 (3.4-60.0)	**0,023**	57.6 (45.2-76.4)	3.5 (1.9-8.2)	**<0.0001**	**<0,0001**	0,13	**<0,0001**
% macrophages	94 (85-97)	69 (37-83)	**0,027**	38 (18-50)	83 (79-95)	**<0.0001**	**<0,0001**	**0,0002**	0,1333
% Lymphocytes	3.4 (2.6-11.0)	2.9 (1.4-9.9)	0,42	2.4 (1.3-4.5)	6.0 (1.6-11.8)	0.22	NA	NA	NA
% eosinophils	0.0 (0.0-0.0)	0.3 (0.0-1.1)	**0,011**	0.2 (0.1-0.9)	0.4 (0.0-1.2)	**0,024**	**0,017**	0,86	**0,04**

Table II: Results are expresses as median (IQR). The variation between the stable, NRAD and fBOS group is calculated with Kruskall Wallis ANOVA and the Mann- Whitney U test is used as post-hoc test for significances of the BOS, NRAD and fBOS group versus the stable group.

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