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Broncho-alveolar lavage fluid recovery correlates with airway neutrophilia in lung transplant patients ☆

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Summary
Broncho-alveolar lavage (BAL) is important to assess airway inflammation. There is debate about the volume instilled, but the variation of BAL fluid recovery (BFR) has received little attention. We investigated the association between BFR and rejection/infection status after lung transplantation (LTx).

We combined clinical findings, FEV_1, transbronchial biopsies and BAL analysis (BFR, interleukin-8 (IL8), cell counts, microbiology) of 115 samples/LTx patients. The patients were divided into 4 groups: stable (subdivided in colonized and non-colonized), acute rejection (AR), Bronchiolitis Obliterans Syndrome (BOS) and infection.

BFR was significantly lower in AR, BOS and infection, and correlated with the severity of AR and BOS. A 10 ml decrease of BFR was associated with a FEV_1 decrease of 4.4% and a %neutrophils and IL8 increase of 9.6% and 9.7 pg/ml, respectively. Colonized stable patients had no significant differences in airway inflammation, FEV_1 and BFR compared to the non-colonized stable patients.

We conclude that a low BFR is an indicator of lung rejection or infection. BFR variation is related to airway obstruction and neutrophilic inflammation, which can cause an increased compliance of the airway wall, making it more collapsible. Airway colonization in stable patients had no effect on airway inflammatory parameters, BFR and FEV_1.

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Introduction

The mechanisms of airway obstruction in acute and chronic rejection after lung transplantation (LTx) are poorly understood. Broncho-alveolar lavage (BAL) is a useful and safe research method for sampling cells and mediators from the upper and lower respiratory tract, to better understand the pathophysiological mechanisms of allograft rejection. Until now, research on the method of lavage was mainly focussed on the volume used for instillation, because of the heterogeneous cell distribution over the different fractions.[1,2] The early fractions represent a bronchial washing and the later fractions rather represent an alveolar washing.[2]

BAL fluid recovery (BFR) variations are, however, only seldom reported. In COPD, BFR is only 10–40% of the instilled volume.[3] No differences were found between BFR of allergic and non-allergic asthmatic children and between infected and non-infected asthmatic children.[4] In LTx, there is scarce data about BFR.[5] It is only briefly mentioned that BFR can be decreased in patients with Bronchiolitis Obliterans Syndrome (BOS).[6,7] Changes in BFR can complicate the interpretation, because a low BFR may predominantly reflect the inflammation in the larger airways.[2] Recently, Lofdahl et al.[8] investigated for the first time more specifically the role of BFR and showed a correlation of BFR with the degree of emphysema. They, however, failed to demonstrate a correlation with FEV_1.

Based on our clinical experience and the article of Lofdahl et al.,[8] the idea was raised to investigate changes in BFR in LTx. LTx recipients who participated in a routine follow-up were included in this retrospective study. BFR and its relation with BAL absolute and differential cell counts, Interleukin-8 (IL8), acute and chronic rejection grade, airway infection and FEV_1 were investigated.

Patients and methods

A total of 152 LTx patients underwent bronchoscopy with BAL in combination with clinical examination, chest X-ray and functional evaluation, as part of a longitudinal follow-up study between October 2001 and December 2004. Routine follow-up bronchoscopies were performed at 28, 90, 180, 360, 540, 720, 900 and 1080 days after LTx, by the same operators in an identical way (LJD and GMV). Transbronchial biopsies (TBB) were taken at days 28 and 90 after LTx and when suspicion of acute rejection (AR) or infection (based on clinical, radiological and pulmonary function criteria). Exclusion criteria were: no BFR recorded, suture problems, post-transplant lymphoproliferative disorder, presence of other malignancies, diffuse alveolar damage or the combination of AR or BOS with another complication at one time point. To eliminate the possibility of multiple sampling, only the last BAL samples of each patient with adequate biopsy and lung function tests was retained. Finally, 4 groups were analyzed: a stable group (no histological or clinical proven AR—no infection on biopsy or clinically suspected—no OB/BOS based on FEV_1, clinical examination or TBB), an AR group (TBB proven and exclusion of other cumulating problems), a BOS group (based on the ISHLT working formulation)[9] and an infection group (based on clinical findings combined with TBB, measurement of blood C-reactive protein (CRP) and exclusion of other cumulating problems). Airway colonization, defined as the presence of positive bacterial/fungal cultures in BAL without evidence of CRP, new radiological infiltrates, fever or need for antibiotic or antifungal treatment, was no exclusion criterion. However, the patients with airway colonization were analyzed separately to identify its effect on the investigated parameters. This study was approved by the Ethics Committee of UZ Gasthuisberg.

FEV_1 was measured with the Masterscreen according to ATS criteria[10] as described before.[11] Bronchoscopy was performed with a bronchoscope (Olympus BF1T30, outer diameter = 5.9 mm and channel diameter = 2.8 mm) under intravenous sedation with 5 mg diazepam. TBB specimens were examined for infection and AR (according to ISHLT guidelines).[12] BAL was performed with two 50 ml aliquots of sterile saline, at room temperature, into the transplanted lung, either in a subsegmental bronchus of the lingula or the right middle lobe. After each 50 ml instilled, the fluid was recovered by gentle manual suction. The reason to use two 50 ml fractions is documented in the discussion. The recovered fractions were pooled and the volume noted. The BAL was analyzed for microbiological, virological, cell counting and IL8 protein. A cytospin (10^5 cells/ml) was stained with May Grünwald Giemsa, and at least 300 cells were counted. IL8 protein was analyzed in BAL supernatant by means of an ELISA (Biosource SA, Nivelle, Belgium; sensitivity = 2 pg/ml). Cell viability is not counted/recorded in our center, as Elssner et al.[13] and Riise et al.[14] demonstrated cell viability not to be associated with the development of BOS in lung transplant patient. Moreover, BAL fluid is kept on ice and immediately processed in the lab.

Statistical analyses were performed with SAS software (version 8.1). All results were presented as median (IQR). In the first part, one-way analysis of variance was used to investigate the variation between the different groups and Bonferroni multiple comparison test was used as post hoc test. Dose–effect relationship was calculated by mean of Spearman rank test. In the final part of our analysis, we calculated the odds of having AR, BOS and/or infection associated with a 1 ml decrease of BFR. For regression analysis non-normally distributed data were log transformed. The first part showed no important variations with log transformation of non-normally distributed data (and accompanying normal statistical tests) and was therefore not shown.

Results

Study protocol

The current study included 152 LTx patients, who underwent a total of 600 bronchoscopic procedures with BAL, together with clinical, biochemical, radiological and pulmonary function evaluation at the moment of bronchoscopy, between October 2001 and December 2004. We excluded 37 patients based on the aforementioned exclusion criteria. One hundred and fifteen patients with 115 BAL samples were divided in a stable, AR, BOS and infection group. Patient's characteristics are given in Table 1.

TBB was available in all 23 patients of the AR group, demonstrating 12 A1, 8 A2 and 3 A3 grade AR. In the other groups, biopsies were not always performed; however, in the stable group 32/52 had a biopsy to exclude AR/infection. The BOS group consisted of: 17 BOS1, 13 BOS2 and 1 BOS3. 20/31 patients with established BOS had a biopsy to confirm the absence of AR/infection. The infection group consisted of 5 patients with CMV pneumonia, 2 with a *Pseudomonas* and 1 with *Escherichia coli* pneumonia and 1 with an acute neutrophilic bronchiolitis. Total and/or differential cell count could not be obtained in 4 samples (1 of the stable group, 1 of the AR group and 2 of the BOS group), due to lysis of the cells and impossibility for interpretation. These patients were retained for analysis as most of the other investigated parameters were available. Smoking cannot be considered as a possible confounding factor as there were within the total study group ($n = 115$) only 4 patients who restarted smoking as demonstrated by positive urine cotinin test; 2 in the stable group; 1 in the AR group and 1 in the infection group.

BAL fluid recovery (BFR)

BFR, expressed as volume of the 100 ml instilled (ml/100 ml), demonstrated significant changes between the 4 groups. BFR of the AR, BOS and infection group were lower compared to the control group (Table 2 and Figure 1A). No differences were found between the AR, BOS and infection group. BFR positively correlated with the degree of airway obstruction measured as FEV_1(%pred.) ($r = 0.24$, $p = 0.0088$, $n = 115$, Figure 1B) and negatively with the BOS grade ($r = -0.54$, $p < 0.0001$, $n = 83$ (BOS group+stable group)) and the AR grade ($r = -0.39$, $p = 0.0005$ and $n = 75$ (AR group+stable group)).

This variation in BFR was not influenced by the different pre-transplant diagnosis as the proportions of the preLTx diagnosis were not different between the study groups. Even more, within the stable group BFR was not different for the different pre-transplant diagnosis (unpublished data).

BAL cellular profile

Total cells were not different between the 3 groups, while absolute ($p = 0.025$) and percentage ($p < 0.0001$) of neutrophils were significantly different between the groups. Both absolute and percentages of neutrophils were higher in BOS patients ($p < 0.05$ and < 0.001, respectively) compared with stable patients (Table 2). The percentages of macrophage demonstrated a significant difference ($p < 0.0001$) by reason of compensating for the variations in neutrophils (Table 2). Absolute lymphocyte counts showed a significant variation ($p = 0.0033$) with an increase in the infection

Table 1 Group characteristics of patients.

Group	Total	Stable	AR	BOS	Infection
n-Value	115	52	23	31	9
POD	245 (60–680)	183 (45–375)	41 (26–208)	1043(639–2151)	176 (90–467)
Age	52(40–57)	50 (39–55)	54 (46–59)	53(33–59)	54 (49–57)
Gender	49F/66M	22F/30M	10F/13M	13F/18M	4F/5M
LTx type					
Bilateral	66	34	14	12	6
Unilateral	38	15	8	13	2
Heart–lung	11	3	1	6	1
Pre-LTx diagnosis					
Emphysma	46	21	9	13	3
PF	24	13	6	3	2
CF	14	8	2	4	0
α_1-ATD	7	1	2	2	2
Eisenmenger	6	2	1	3	0
PPH	5	1	1	3	0
Miscellaneous	13	6	2	3	2
Medication					
FK506	89	37	14	29	9
CsA	26	15	9	2	0
MMF	35	13	5	15	2
AZA	68	32	17	12	7
Methylprednisolone	84	52	23	31	9
Rapamycin	5	0	0	5	0

POD = post-operative day, LTx = lung transplantation, PF = pulmonary fibrosis, CF = cystic fibrosis, α_1-ATD = α_1-anti-trypsin deficiency, PPH = primary pulmonary hypertension, miscellaneous = bronchiectasis (5), retransplantation for BOS (3), Lymfangioleiomyomatosis (1), obliterative bronchiolitis (1), asbestosis (1), sarcoidosis (1), kartagener (1). FK506 = tacrolimus, CsA = cyclosporin A, MMF = mycofenolate mofetil, AZA = azathioprine.

Table 2 BFR, FEV_1, cell differentials and IL8 levels.

Group	Stable	AR	BOS	Infection	One-way analysis of variance
FEV_1 (% predicted)	79(53–98)	46(38–65)***	56(40–62)	72(38–94)	ND
BFR (ml/100 ml)	46(40–55)	38(24–47)**	35(26–40)***	30(23–45)**	<0.0001
Total cells ($\times 10^3$/ml)	140(98–274)	236(124–403)	179(60–369)	367(241–509)	0.16
Macrophages (%)	88(83–94)	84(47–93)	66(30–90)***	45(34–53)***	<0.001
Macrophages ($\times 10^3$/ml)	124(68–217)	169(66–230)	78(48–207)	142(73–299)	0.29
Neutrophils (%)	2.5(1.5–5.8)	8.0(1.8–49.5)	15.0(2.8–64.3)***	46.5(20.7–55.0)**	<0.0001
Neutrophils ($\times 10^3$/ml)	2.7(1.1–11.5)	12.1(4.0–136.9)	12.2(3.6–231.5)*	134.1(53.4–315.9)	0.025
Lymphocytes (%)	6.5(2.8–11.0)	5.0(2.5–11.0)	5.3(2.0–9.3)	8.8(3.5–17.4)	0.73
Lymphocytes ($\times 10^3$/ml)	9.4(3.1–24.0)	15.2(5.2–26.1)	7.1(2.6–23.0)	23.8(13.7–87.2)**	0.0033
Eosinophils (%)	0.0(0.0–0.0)	0.0(0.0–0.0)	0.0(0.0–0.5)	0.2(0.0–2.6)	ND
Eosinophils ($\times 10^3$/ml)	0.0(0.0–0.0)	0.0(0.0–0.0)	0.0(0.0–0.7)	0.6(0.0–5.6)	ND
IL8 protein (pg/ml)	23(11–84)	50(16–181)	70(23–247)*	141(54–397)*	0.0097

Results are expressed as median (IQR) and statistically significant variation is calculated with one-way analysis of variance. ND = not determined. Bonferroni's multiple comparison test is used as post hoc test for significances of the AR, BOS and infection group versus the stable group. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

group compared to the stable group ($p<0.01$, Table 2). Eosinophils were neglectable (a median value under 1%). BFR was negatively correlated with the numbers of neutrophils, both expressed as a percentage ($r=-0.42$, $p<0.0001$, $n=113$, Figure 1C) and as absolute number ($r=-0.40$, $p<0.0001$, $n=111$). Neutrophil percentages showed a significant negative correlation with FEV_1 ($r=-0.31$, $p=0.0009$, $n=113$, Figure 1E).

Analysis of IL8 protein in BAL

IL8 demonstrated significant variations between the 4 groups ($p=0.0097$). IL8 was increased in patients with BOS ($p<0.05$) or with infection ($p<0.05$) compared with the stable group. BFR correlated negatively with IL8 ($p=-0.37$, $p<0.0001$, $n=115$, Figure 1D) and with the number of BAL neutrophils ($p=-0.45$, $p<0.0001$, $n=113$).

Prognostic power of BFR

Logistic regression performed on all samples demonstrated that, in comparison with the stable group, a decrease of 1 ml of BFR was associated with 9.2% more chance of having a complication like AR, BOS or infection. Analysis of the stable and BOS group alone demonstrated that, for a 1 ml decrease of BFR, the odds ratio of having BOS was 1.134. In the AR group the risk of having AR increased with 6.8% and in the infection group the risk increased with 10.6% with each ml less BFR compared to the stable group (Table 3). Performing stepwise regression analysis on the complete study population demonstrated that a decrease of BFR with 10 ml resulted in a significant decrease of the FEV_1(%pred.) with 4.43% and a significant increase of %neutrophils with 9.56% and IL8 protein with 9.67 pg/ml. We adjusted for immunosuppressive therapy (cyclosporine/tacrolimus and azathioprine/mycophenolate mofetil), and type of transplantation in the analysis of FEV_1. In the analysis for neutrophils and IL8 we adjusted for age, gender, type of transplantation and therapy (Table 3).

The effect of airway colonization in the stable group

To investigate the possible influence of airway colonization, the stable group ($n=52$) was subdivided according to the presence of organisms in the BAL. The infection group from the first part is used as a reference. Colonization was present in 24 samples and consisted of CMV ($n=11$), *Pseudomonas aeruginosa* ($n=6$), *Aspergillus fumigatus* ($n=5$), *Candida albicans* ($n=2$), coagulase-negative *Staphylococcus* ($n=1$), *Aspergillus niger* ($n=1$), *Alcaligenes xylosoxidans* ($n=1$), *E. coli* ($n=1$), *Streptococcus penumoniae* ($n=1$), *Proteus mirabilis* ($n=1$), *Serratia marcescens* ($n=1$) and *Stenotrophomonas maltophilia* ($n=1$). Nine samples demonstrated a combined colonization. Variations were found for IL8 ($p=0.0051$), total number and percentage of neutrophils ($p=0.0037$, $p<0.0001$, respectively), total number of lymphocytes ($p=0.017$), total number of inflammatory cells ($p=0.043$) and total number of macrophages ($p<0.0001$). Eosinophils were not further investigated as levels were negligible. Significant differences also were found for CRP ($p<0.0001$) and BFR ($p=0.017$) but not for FEV_1(%pred.) ($p=0.61$) (Figure 2). However, all the variations were caused by the infection group. Bonferroni post hoc test showed no difference between non-colonization and colonization samples of the control group.

Discussion

In the present study, we evaluated whether BFR is related to rejection or infection in LTx patients. BFR was significantly decreased in LTx patients with infection or rejection (acute or chronic). We also demonstrated the BFR to reflect

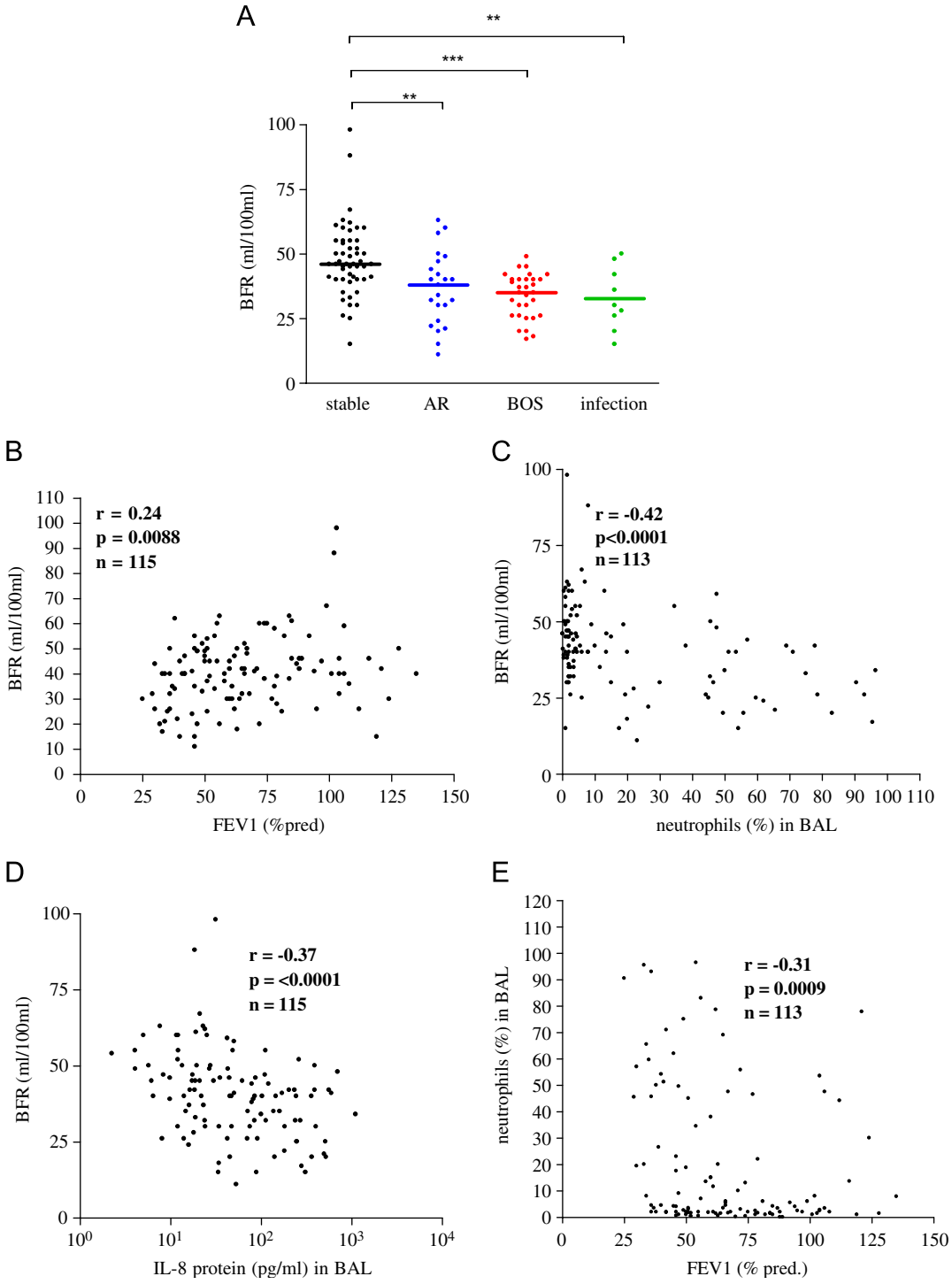

Figure 1 BAL was performed in 115 LTx patients by instillation of two 50 ml sterile saline aliquots. Results are expressed as volume of BFR of the 100 ml instilled (ml/100 ml). (A) One-way analysis of variance demonstrated significant variations between the groups ($p<0.0001$). BFR was significantly decreased in patients with AR ($p<0.01$, $n=23$), BOS ($p<0.001$, $n=31$) and infection ($p<0.01$, $n=9$) compared to stable LTx patients ($n=58$). **$p<0.01$ and ***$p<0.001$. (B) Spearman correlation between FEV_1 (%pred.) and BFR of all patients ($n=115$). (C) Spearman correlation between the percentage of neutrophils in BAL and BFR of $n=113$ patients. (D) Spearman correlation between IL8 (pg/ml) protein in BAL and BFR of all patients ($n=115$). (E) Correlation between the percentage neutrophils in BAL and FEV_1 (%pred.) of all patients ($n=113$). *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

the degree of airway obstruction (FEV_1) and the inflammation in the lung (BAL neutrophilia and IL8). Each 1 ml decrease in BFR gives around 9.2% more risk of having lung rejection (BOS or AR) or infection. This is accompanied by a significant variation in FEV_1(%pred.), %neutrophils and IL8 protein in BAL. Airway colonization has always been a problem in transplantation, as we have no idea whether the (asymptomatic) presence of an organism in the

Table 3 Logistic and single-regression analysis for variation of BFR.

Logistic regression analysis (Odds ratio associated with AR and/or BOS if BFR decreases with 1 ml)	Odds ratio	95% Confidence interval	*p*-Value
BOS or AR or infection (whole group)	1.092	1.049–1.138	<0.0001
BOS (AR and infection group not included)	1.134	1.063–1.211	0.0002
AR (BOS and infection group not included)	1.068	1.019–1.119	0.0061
Infection (AR and BOS group not included)	1.106	1.017–1.203	0.018
Single-regression analysis (changes if BFR decreases with 10 ml)	**Variation**	**95% Confidence interval**	***p*-Value**
FEV_1 (%pred.)*	−4.43	−1.37 to −7.49	0.0054
%Neutrophils in BAL†	9.56	9.36–9.72	<0.0001
IL8 protein (pg/ml) in BAL†	9.67	9.48–9.87	0.0015

CsA = cyclosporine; FK506 = tacrolimus; AZA = azathioprine; MMF = mycophenolate mofetil.
*We adjusted for therapy (CsA/FK506 and AZA/MMF) and type of transplantation.
†We adjusted for age gender, therapy (CsA/FK506 and AZA/MMF) and type of transplantation.

airway influences its inflammatory status. The present study documents that colonization, at least in stable LTX patients, did not have any significant impact on the lung function, airway inflammation (neutrophils, IL8), blood CRP and BFR.

BAL is a generally accepted clinical and research tool, to assess inflammation of the airways in different patient populations, including LTx patients. Technical procedures of BAL were evaluated in the late 1990s by an ERS task force[3] and recommended to use 5 aliquots of 50 ml. The first was designated as the bronchial fraction and the latter as the alveolar fractions. This first bronchial fraction was recommended to be discarded and analysis should be performed on the other fractions, which were pooled. The article of Martin et al. demonstrated, in patients with chronic bronchitis, that cell profiles vary upon the volume used for BAL. Neutrophils were predominantly present in the early fractions and macrophages in the later fractions.[2] The importance of using standardized volumes and aliquots for BAL sampling is now generally accepted. This is, however, a problem in patients with severe lung diseases (like COPD and LTx), as described in the article of Lofdahl et al.[8] Severely diseased lungs simply do not tolerate high volumes. As a consequence, performing BAL already presents a problem regarding the instillation volume. Using too little volume, raises no problems as regard to instillation and recovery, but only represents a bronchial lavage (BL). Using a higher volume (combined with discarding the first fraction) rather represents an alveolar lavage (AL), which may be problematic with respect to recovery and later analysis, not withstanding the possible clinical problems in very sick patients. Our decision to use 100 ml in 2 fractions of 50 ml, and to pool the fractions, is a result of this, although this is not in accordance with the ERS Task force guidelines. LTx patients simply do not always tolerate instillation of higher volumes. Not discarding the first (bronchial) fraction of the BAL was based on the potential influence of the bronchial inflammation in the onset of rejection and/or infection, which must not be underestimated in LTx patients.

Until now, the LTx literature only discussed the volume used for BAL.[1] The importance of BFR was never discussed, only sometimes mentioned[6,7] or speculated on.[15] In COPD (where the major sites for airflow limitation are located in peripheral airways and parenchyma), BFR volumes were significantly lower compared to smoking control subjects with normal lung function.[16] Lofdahl et al.[8] stipulated that this decrease makes BAL difficult to interpret, as it may reflect larger airways rather than the alveolar compartment. It would be tempting to eliminate all BAL samples with a return below a certain volume (<30 ml). This can bias the results as most of the eliminated samples are from the more diseased patients, which is confirmed by the correlations between BFR and parameters like neutrophilia, IL8 level, degree of BOS and FEV_1. Consequently, there is the dilemma of underestimating the airway inflammation (loss of the more severely diseased patients), on the one hand, or overestimating the inflammation (a lower BFR reflects more BL containing more neutrophils), on the other hand. In our study, no BAL samples were eliminated for reasons of a low (<30 ml) BFR, but, as a consequence, we may have found more severe neutrophilic inflammation.

Neutrophils are increased in some types of infection, in BOS,[17] at least in a subgroup,[18] but also in AR.[11] However, even very high numbers of airway neutrophils may be found in stable, AR and BOS samples. It would be tempting to explain this by the presence of a secondary infection; however, in our study this possible explanation was eliminated based on clinical, bacteriological and histopathological findings. High numbers of airway neutrophils are not abnormal as it has also been demonstrated, although not consistently, in several other studies.[19–21] These papers clearly illustrate that BAL neutrophils may be present even in stable patients or in patients with an AR or BOS. A nice example is the article by Slebos et al.[5] Although no infection is present and the median BAL neutrophil percentages in stable, AR and BOS samples, were only 2%, 3% and 9% in both the bronchial and the alveolar fraction, the neutrophil percentages go up to 73%, 89% and 97%, respectively.[3] These

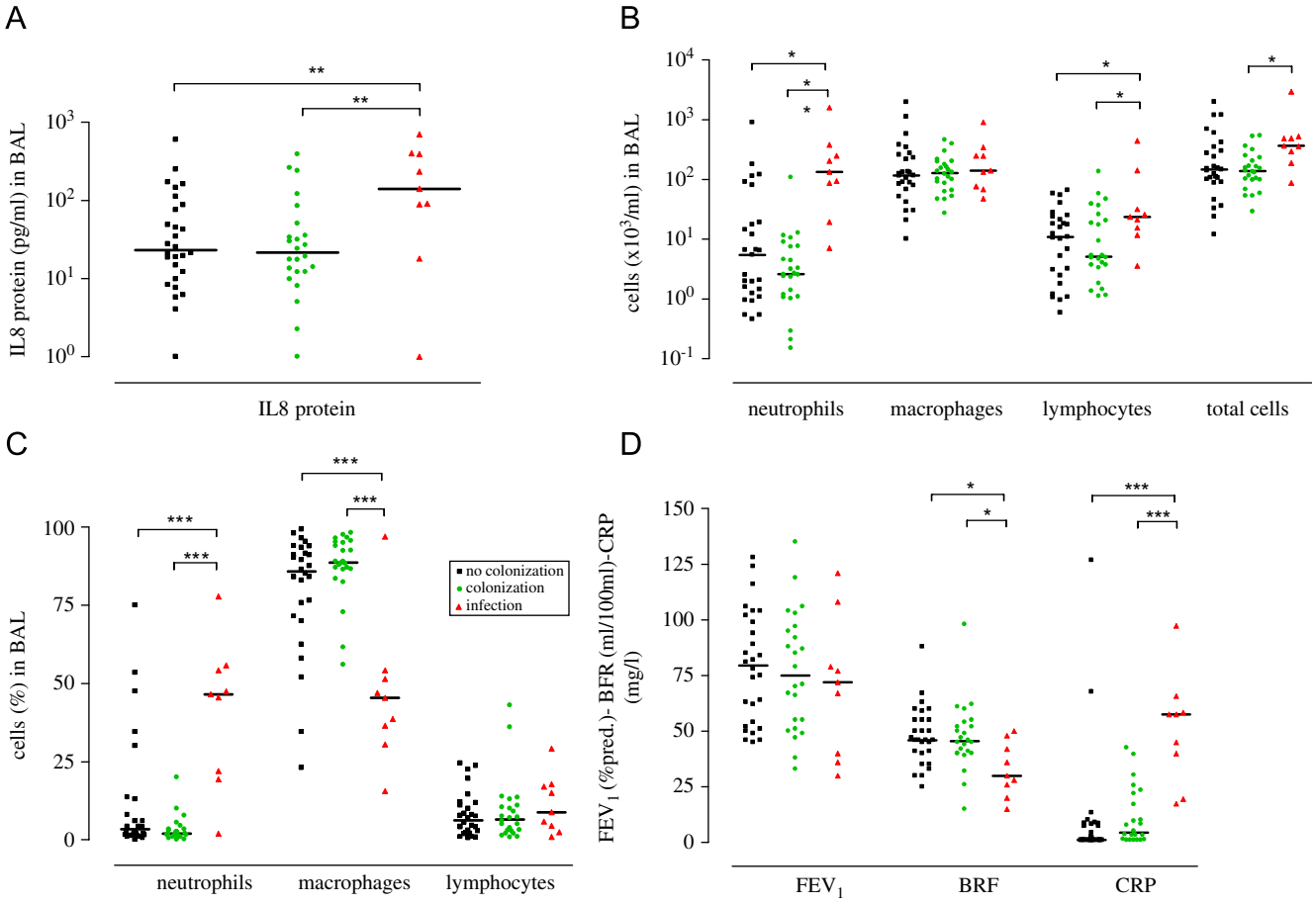

Figure 2 The stable group (52 stable samples/patients) was divided in 2 subgroups based on the presence or absence of colonization. Various types of colonization were demonstrated in 24 samples and no colonization could be demonstrated in 28 samples. The infection group included 9 patients. Bonferroni post hoc test revealed no significant difference between the colonized and non-colonized group for all the different parameters. Only the infection group demonstrated clear increases compared to both the colonized and non-colonized group for the different parameters. (A) IL8 protein, (B) cells numbers in BAL, (C) percentage of cells in BAL, (D) FEV_1 (%pred.), CRP and BFR (ml/100 ml). *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

studies clearly indicate, as our own study, that neutrophil numbers can sometimes be very high but not necessarily relate to an infection as is sometime postulated.[7] These varying percentages may also partly be explained by the method of performing BAL as discussed earlier.

Airway colonization could represent another possible explanation for the high BAL neutrophilia. Some authors even eliminate samples from their analysis when they are colonized as little is known on its possible relevance.[7] However, in this study the presence of colonization in the stable group clearly demonstrates not to affect airway (BAL) neutrophilia or any other inflammatory cell type. This was confirmed by Ward et al.,[22] who also found no difference in airway inflammatory parameters between BAL samples with bacteria or CMV present, compared to absence of organisms. Therefore, it seems no longer necessary to exclude colonized samples when there is no clinical, radiological or pathological evidence for infection. There are, however, 2 remarks to be made. The definition of colonization can be a matter of debate. We defined colonization as the presence of an organism within the analyzed BAL sample, without evidence for infection as defined earlier. We did not define colonization as repeated isolation of the species from BAL culture and/or repeatedly increased antibody levels in the blood. The other possible shortcoming is that the colonized group consisted of a mixture of several kinds of bacteria, fungi and viruses. We can, therefore, not exclude that colonization of some type of bacteria can induce inflammation or even influence the development of BOS, as we have recently demonstrated for Pseudomonas and Pseudomonas-like bacteria.[23,24]

Logistic regression analysis was performed to determine an increase in risk of rejection (BOS/AR) or infection when there is a decrease of BFR. A 9.2% higher risk was found for having rejection (BOS/AR) or infection, if BFR decreases with only 1 ml, what makes it a very interesting tool in the follow-up of the patients. Small changes were found between AR, BOS and infection, respectively 6.8%, 13.4% and 10.6. It is therefore clear that BFR itself can already give some indication towards AR, BOS or infection and may demand increased vigilance. It was also remarkable that a 10 ml decrease of BFR is accompanied by an increase of neutrophils with 9.5% and IL8 with 10.4 pg/ml and a decrease of the FEV_1(%pred.) with 4.5%, which again

indicates that lung function and inflammation are related to each other and that this is reflected in BFR.

The underlying mechanism that results in a decreased BFR remains speculative but may be explained by the smaller airway space (diameter) of the bronchioli and by the loss of rigidity (increased compliance) of the airway wall, which are caused by inflammation. Inflammatory cells and markers (IL8) were investigated in order to have more information on the mechanisms of this increased compliance. The key element seems to be the increased airway neutrophilia and related IL8 chemoattractant,[13,17] which was found during rejection and infection. It seems acceptable to postulate that airway neutrophilia increases the collapsibility leading to reduced BFR and a FEV_1 decrease. It is tempting to speculate whether a lower BFR is related with increased neutrophilia/IL8 and so can be associated with azithromycin responsiveness. Therefore, in our center when the BFR starts to decrease we are alerted (not more not less) for the lavage results (FEV_1/BOS, neutrophilia, IL8) keeping in mind that an increased neutrophlia/IL8 is a predictive factor for the responsiveness of azithromycin.[18,25]

A possible limitation of the study can be the lack of a healthy control group of asymptomatic non-smokers about the same age ($\pm$52 years). Especially as there is some contradictory data regarding BFR between healthy individuals and stable LTx patients. On the one hand, Ward et al.[6] and Zheng et al.[7] reported a significant decrease of BFR in stable LTx patients versus healthy individuals, but on the other hand, an other report by Zheng et al.[26] found no difference between healthy individuals and stable LTx patients, although each time 3 aliqouts of 60 ml were used. Martin et al.[2] demonstrated the BFR to be lower in chronic bronchitis patients compared to healthy individuals in the later (alveolar) fraction and not in the early (bronchial fractions). This was confirmed in LTx by Slebos et al.[5] where the BFR was lower in the alveolar fraction of patients with BOS compared to stable patients but not in the bronchial fraction.

In conclusion, the present results indicate that in LTx a low BFR may predict the ongoing development of AR, BOS or infection. A reduced BFR also correlates with the FEV_1, and the inflammation in the airways (IL8 and neutrophils) and speculates about azithromycin responsiveness. The correlation between the decreased BFR and the increased neutrophilic inflammation may indicate that neutrophils are the cause of this increased compliance of the airway wall. As a result, a low BFR may demand a more intense vigilance for rejection and infection in LTx patients. These data are not influenced by colonization of the airways.

Conflict of Interest

None of the authors have a conflict of interest to declare in relation to this work.

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