

## Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease

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*Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. N. Soler, S. Ewig, A. Torres, X. Filella, J. Gonzalez, A. Zaubet. © ERS Journals Ltd 1999.*

**ABSTRACT:** The effect of bacterial colonization of the bronchi on the progress of airflow limitation is not well known. Therefore, the pattern of airway inflammation in smokers and patients with stable chronic obstructive pulmonary disease (COPD) and its relation to bronchial microbial colonization was assessed.

Eight nonsmoking and 18 smoking controls as well as 52 patients with COPD (28 mild, 11 moderate and 13 severe) were studied. All subjects were investigated by means of flexible bronchoscopy including protected specimen brush and bronchoalveolar lavage (BAL) sampling. Differential cell counts, cytokine (interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-10 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) concentrations and microbial patterns were determined in BAL fluid.

Forced expiratory volume in one second (FEV<sub>1</sub>) % of the predicted value was inversely correlated with pack-yr of cigarette smoking ( $r=-0.47$ ,  $p<0.0001$ ), the percentage of neutrophil ( $\rho=-0.56$ ,  $p<0.0001$ ) and IL-6 ( $\rho=-0.37$ ,  $p=0.01$ ) and IL-8 concentration ( $\rho=-0.43$ ,  $p=0.004$ ) in BAL fluid. Accordingly, pk-yr of cigarette smoking ( $\rho=0.39$ ,  $p=0.01$ ) and IL-8 ( $\rho=0.69$ ,  $p<0.0001$ ) and TNF $\alpha$  ( $\rho=0.4$ ,  $p<0.005$ ) were positively correlated with the percentage of neutrophils in BAL fluid. Smoking controls and COPD patients were mainly colonized in the bronchial tree (33%) by community endogenous potentially pathogenic micro-organisms (PPMs). Colonization rates and patterns of PPMs were not affected by severity of airflow obstruction. The presence of PPMs was significantly associated with higher percentages of neutrophils ( $33.2\pm 10.4\%$  versus  $10.1\pm 3.5\%$ ,  $p=0.02$ ) and TNF- $\alpha$  concentration ( $29.9\pm 10.8$  versus  $6.3\pm 2.1$  pg·mL<sup>-1</sup>,  $p=0.01$ ) in BAL fluid.

In conclusion, bronchial neutrophilia is a key inflammatory pattern in chronic obstructive pulmonary disease patients. Bronchial colonization with potentially pathogenic micro-organisms may represent an independent stimulus for additional airway inflammation.

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Bacterial colonization of distal airways with pathogenic as well as nonpathogenic agents is frequently found [1–4]. The significance of bacterial colonization of the bronchial tree in terms of the development and progression of airflow limitation as well as in acute exacerbations is not precisely known. Nevertheless, there is evidence that the acute and chronic inflammatory response to bacterial pathogens is an important contributor to the damage of epithelial surfaces, thus promoting further bronchial colonization, acute infections and impairment of local immune responses [5–8].

Nevertheless, the exact relation between markers of bronchial airway inflammation and smoking behaviour, severity of airway obstruction and microbial patterns of bronchial colonization has not yet been established. Particularly, no study to date has concomitantly assessed inflammatory as well as microbial patterns by means of bronchoscopically retrieved samples in patients with severe chronic obstructive pulmonary disease (COPD).

Therefore, a study was carried out in patients with stable COPD of differing severity, without prior antimicrobial

treatment, in order to determine the pattern of bronchial airway inflammation *via* differential cell count and cytokine patterns found in bronchoalveolar lavage (BAL) fluid and their relationship with microbial patterns of bronchial colonization assessed by the protected specimen brush technique (PSB) and BAL.

### Materials and methods

#### Patient population

The study was conducted between January 1996 and March 1997. Stable nonsmoking patients, smoking patients with normal spirometry results, and stable COPD patients with an indication for bronchoscopy were eligible. A stable clinical condition was defined by the absence of symptoms of a lower respiratory tract infection (increase in dyspnoea, cough and/or purulent sputum) for  $\geq 3$  months prior to inclusion in the study. Exclusion criteria were: antimicrobial treatment during the last  $\geq 4$  weeks, treatment with oral

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corticosteroids during the last 3 months, hospital admission during the last 3 months, evidence of bronchiectasis according to clinical and chest radiography criteria and any severe comorbid conditions such as severe immunosuppression, malignancies, and coagulopathies.

Eight nonsmoking and 18 smoking patients without COPD comprised the control groups, and 52 patients with COPD the case groups. The indications for performing fiberoptic bronchoscopy were: mild haemoptysis (n=40), hilar enlargement (n=18), pulmonary nodules (n=10) and others (n=10). Exploration of the bronchial tree and further evaluation showed no evidence for neoplasia or sarcoidosis in any case.

Smoking controls had a history of cigarette smoking of  $\geq 15$  pack-yrs (range 15–80, mean $\pm$ SD 41 $\pm$ 21). World Health Organization criteria for chronic bronchitis (cough and sputum for  $\geq 3$  months during the last 2 yrs) were met by nine (50%) smoking controls and 28 (54%) COPD patients. All patients underwent forced spirometry as well as reversibility testing. No patient fulfilled reversibility criteria as defined by the American Thoracic Society (ATS) guidelines [9]. COPD case groups were classified as mild forced expiratory volume in one second (FEV1)  $>50\%$  of the predicted value, n=28), moderate (50–35%, n=11), and severe ( $<35\%$  pred, n=13) according to ATS guidelines [9]. No control and no COPD patient had evidence of atopy or asthma. Patients with severe COPD significantly more often had a history of prior acute exacerbations within the last year as compared to patients with mild and moderate COPD ( $p<0.0001$ ) (table 1).

#### Study protocol

The study protocol was approved by the local ethical committee. Informed written consent was obtained from all patients.

The history of current symptoms, number of prior exacerbations, concomitant morbidities, and current treatment were retrieved by means of a standardized questionnaire.

All patients were subjected to fiberoptic bronchoscopy including the PSB technique and BAL. Flexible bronchoscopy was performed transnasally (BF 30; Olympus, New

Hyde Park, NY, USA). Prior to bronchoscopy, patients received topical anaesthesia by inhalation of nebulized lignocaine (8 ml, 4% solution). No additional anaesthetic was instilled nor was any aspiration performed through the inner channel of the bronchoscope during the procedure. After inspection of the bronchial tree, a PSB (Microbiology brush; Mill-Rose Laboratory Inc., Mentor, OH, USA) sample was obtained from a segmental orifice of the right or left lower lobe. BAL was performed at the same site with  $5 \times 30$  mL nonbacteriostatic saline. The first reaspirated portion was discarded. The volume of the remaining recovered BAL fluid was recorded, and then divided into two equal aliquots for cytological examination/cytokine measurements and quantitative bacterial cultures.

#### Cytological examinations

The BAL fluid was centrifuged for 10 min at 500 revolutions per minute (Cytospin II, Shandon). The cell-free supernatants were stored at  $-70^\circ\text{C}$  until analysed. Cell viability as well as total and differential cell counts were determined as described elsewhere [10].

#### Cytokine measurements

The concentrations of the following cytokines in BAL fluid supernatant were determined: (IL)-1 $\beta$ , IL-6, IL-8, IL-10, and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). A solid phase enzyme-linked immunosorbent assay (ELISA) based on the quantitative immunometric sandwich enzyme immunoassay technique in a microtitre plate (Enzyme Amplified Sensitivity immunoassay (EASIA); Medgenix Diagnostics, Fleurus, Belgium (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) and Perseptive, Framingham, MA, USA (IL-8 and IL-10)) was used. This ELISA method used a monoclonal antibody specific to the particular cytokine to be analysed coated on the microtitre plate to create the solid phase. BAL fluid specimens were pipetted into the wells in duplicate. After the cytokine had bound to the immobilized antibody, a second monoclonal antibody was added to the wells and allowed to bind to a different epitope on the same cytokine. In the assays for IL-1 $\beta$ , IL6 and TNF- $\alpha$ , the monoclonal antibody was conjugated to horseradish peroxidase. In the

Table 1. – Characteristics of controls and patients with chronic obstructive pulmonary disease (COPD)

	Control		COPD		
	Nonsmoker	Smoker	Mild	Moderate	Severe
Subject n	8	18	28	11	13
Age yrs **	51 $\pm$ 11	55 $\pm$ 9	64 $\pm$ 11	66 $\pm$ 10	68 $\pm$ 9
Sex M/F*	5/3	13/5	27/1	11/0	13/0
Smoking history					
current/past <sup>§</sup>	–	18/0	14/14	2/9	2/11
pack-yrs	–	41 $\pm$ 21	46 $\pm$ 21	51 $\pm$ 19	60 $\pm$ 17
FEV1 % pred***	95 $\pm$ 7	94 $\pm$ 10	65 $\pm$ 9	43 $\pm$ 5	28 $\pm$ 6
Chronic bronchitis	–	9 (50)	13 (46)	6 (55)	9 (69)
History of acute exacerbations*					
None	–	–	26	7	2
1	–	–	1	3	6
>1	–	–	1	2	5
Inhaled steroid treatment	–	–	6	6	9

Data are presented as mean $\pm$ SD or n with or without percentage in parentheses. <sup>§</sup>: smoking cessation  $\geq 2$  yrs. The Chi-squared test was used to compare categorical variables and analysis of variance (ANOVA) continuous variables between the two groups, control and COPD. M: male; F: female; FEV1: forced expiratory volume in one second. \*:  $p<0.01$  (Chi-squared test); \*\*:  $p<0.01$  (ANOVA); \*\*\*:  $p<0.0001$  (ANOVA).

assays for IL-8 and IL-10, a biotinylated antibody directed against the cytokine was added. Excess antibody was removed by washing and enzyme-conjugated streptavidin, which binds to the sandwich cytokine monoclonal antibody, was then added. In both types of assay, after washing away any unreacted conjugate, tetramethylbenzidine (substrate) was added, and colour developed in proportion to the amount of the particular cytokine bound to the plate. Colour development was stopped by stop solutions and colorimetric determination was performed using a reader EASIA-reader (Medgenix Diagnostics). The concentrations of cytokines in the samples were determined by comparing the optical densities of the samples to those on standard curves. Results were expressed in pg·mL BAL fluid<sup>-1</sup>. The sensitivity of the technique allowed the detection of levels as low as 1 pg·mL<sup>-1</sup> for IL-8 and IL-10, 2 pg·mL<sup>-1</sup> for IL-1 $\beta$ , and 3 pg·mL<sup>-1</sup> for IL-6 and TNF- $\alpha$ .

The levels of cytokines were given as the concentration, in the BAL fluid because the instilled volume was always the same and no reference molecule (urea or albumin) compensating for the dilution of the lavaged fluid is valid [11].

#### Microbiological evaluation

Respiratory samples were Gram-stained and homogenized. Undiluted as well as diluted secretions were plated on blood, chocolate, Wilkens-Chalgren and Sabouraud agar. Identification of micro-organisms was performed according to standard methods [12]. Susceptibility testing was performed using the agar diffusion method. Results of quantitative cultures were expressed in colony-forming units (cfu).

Bacterial agents were classified into potentially pathogenic microorganisms (PPMs) or non-PPMs as described elsewhere [13]. In contrast to in pneumonia, there is no accepted standard of microbial quantification for defining colonization. In order to minimize the risk of dealing with contaminants, only pathogens with counts of  $\geq 10^2$  cfu·mL<sup>-1</sup> in PSB samples and  $\geq 10^3$  cfu·mL<sup>-1</sup> in BALF were regarded as significant.

#### Statistical analysis

Data are presented as means $\pm$ SEM unless stated otherwise. Continuous variables were compared using the non-parametric Mann-Whitney U-test and categorical variables

using the Chi-squared test or Fisher's exact test, where appropriate. Unifactorial analysis of variance with *post hoc* Bonferroni correction was performed for multiple comparisons of continuous variables. Correlations were calculated using Spearman's rank test. All reported p-values are two-tailed. The level of significance was set at 5%.

## Results

#### Patients

Seventy-eight patients were included in the study. Bronchoscopy was well tolerated by all patients without adverse effects. Patient characteristics are given in table 1.

#### Number of samples retrieved

Twelve controls (all smokers) and all COPD patients underwent quantitative culture of PSB samples, and quantitative cultures of BALF were performed in nine controls (all smokers) and in 24 patients with mild, eight with moderate and 11 severe with COPD. Differential cell counts in BALF were performed in all nonsmoking controls, 10 smoking controls and in 14 patients with mild, six with moderate and 10 with severe COPD. Cytokine patterns in BALF were determined in all nonsmoking controls, 10 smoking controls and in 13 patients with mild, seven with moderate and 10 with severe COPD.

#### Total and differential cell counts in bronchoalveolar lavage fluid

The recovery of BAL fluid was significantly greater in nonsmoking controls than in patients with COPD (mean $\pm$ sd 64 $\pm$ 21 mL BAL fluid in nonsmoking controls, 44 $\pm$ 10 mL in smoking controls, 35 $\pm$ 21 mL in patients with mild COPD, 26 $\pm$ 19 mL moderate COPD, and 21 $\pm$ 11 mL severe COPD;  $p < 0.0001$ ). There were no significant differences in cell viability and total cell counts in BAL fluid between the groups. However, there was a trend towards greater cellularity in smoking controls and COPD patients as compared to nonsmoking controls (table 2).

The mean total and differential cell counts are given in table 2. There was a trend for the percentage of neutrophils to be higher in smoking controls and mild and moderate COPD patients as compared to nonsmoking

Table 2. – Cell viability and total and differential cell counts in bronchoalveolar lavage fluid from controls and chronic obstructive pulmonary disease (COPD) patients

	Control		COPD		
	Nonsmoker	Smoker	Mild	Moderate	Severe
Subjects n	8	10	14	6	10
Cell viability %	85.8 $\pm$ 3.8	77.5 $\pm$ 6.1	83.0 $\pm$ 2.6	87.7 $\pm$ 1.5	73.6 $\pm$ 4.1
Total cell count 10 <sup>5</sup> cells·mL <sup>-1</sup>	2.1 $\pm$ 0.5	5.9 $\pm$ 1.4	13.3 $\pm$ 4.2	7.2 $\pm$ 1.5	6.6 $\pm$ 1.9
Macrophages %	87.1 $\pm$ 2.1	83.5 $\pm$ 8.8	77.4 $\pm$ 5.8	81.0 $\pm$ 8.9	65.1 $\pm$ 9.3
Neutrophils %	1.3 $\pm$ 0.5	11.4 $\pm$ 8.8	13.2 $\pm$ 6.2	11.7 $\pm$ 6.2	26.7 $\pm$ 9.9
Lymphocytes %	11.6 $\pm$ 1.8	5.0 $\pm$ 1.6	8.6 $\pm$ 1.8	4.5 $\pm$ 1.4	11.7 $\pm$ 2.7
Eosinophils %	0	0.1 $\pm$ 0.1	0.8 $\pm$ 0.5	2.5 $\pm$ 1.9	0.3 $\pm$ 0.3
Macrophages 10 <sup>5</sup> cells·mL <sup>-1</sup>	1.8 $\pm$ 0.47	5.1 $\pm$ 1.4	10.8 $\pm$ 3.7	5.6 $\pm$ 1.4	2.8 $\pm$ 0.6
Neutrophils 10 <sup>5</sup> cells·mL <sup>-1</sup>	0.03 $\pm$ 0.01	0.55 $\pm$ 0.44	1.52 $\pm$ 1.8	0.95 $\pm$ 0.6	3.3 $\pm$ 1.8
Lymphocytes 10 <sup>5</sup> cells·mL <sup>-1</sup>	0.24 $\pm$ 0.07	0.36 $\pm$ 0.18	0.89 $\pm$ 0.26	0.39 $\pm$ 0.14	0.78 $\pm$ 0.32
Eosinophils 10 <sup>5</sup> cells·mL <sup>-1</sup>	0	0.01 $\pm$ 0.01	0.03 $\pm$ 0.02	0.24 $\pm$ 0.19	0.03 $\pm$ 0.03

Data are presented as mean $\pm$ SD. No differences were statistically significant.

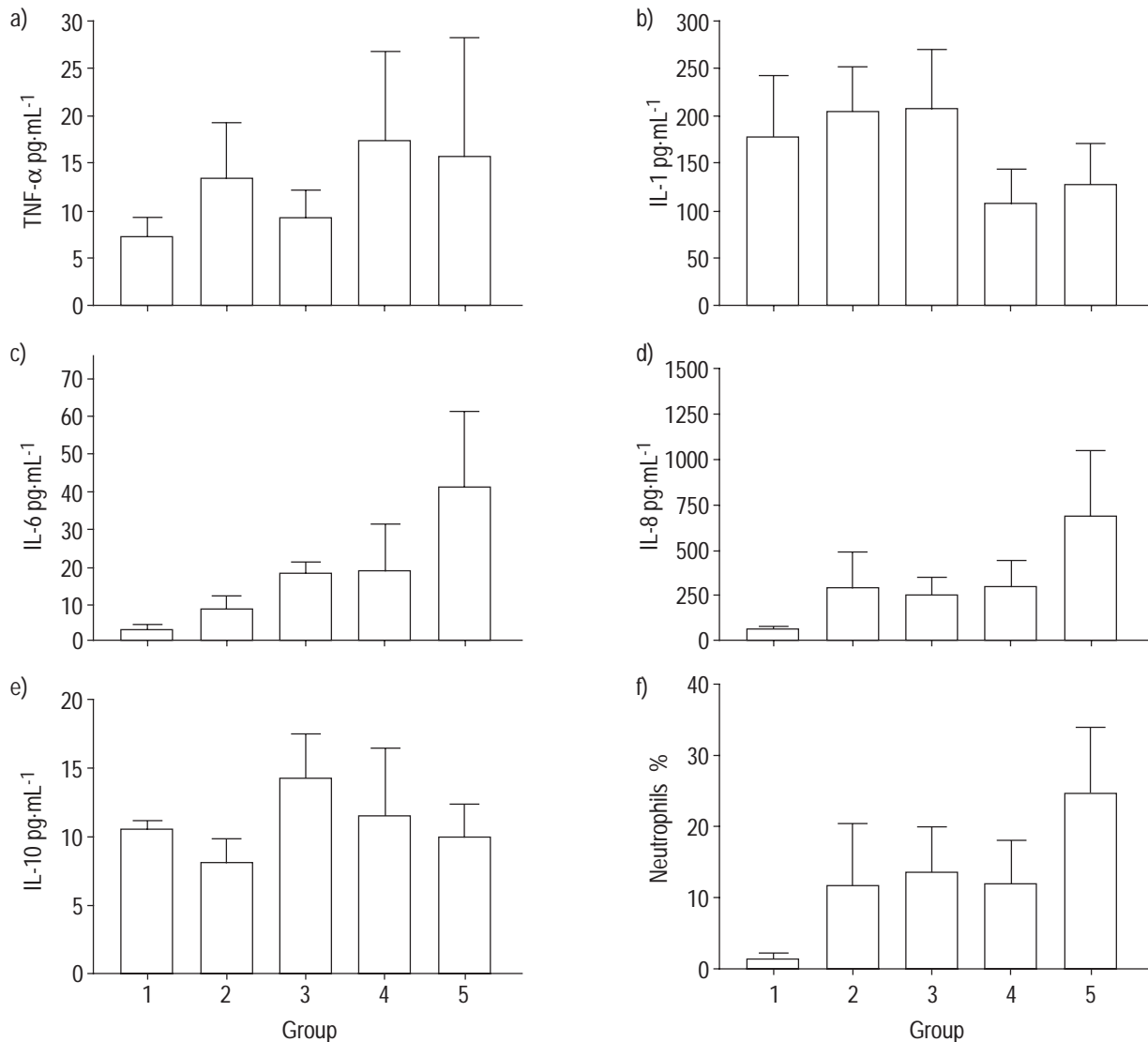


Fig. 1. – Cytokine levels and neutrophil counts in bronchoalveolar lavage fluid (BALF): a) tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ); b) interleukin (IL)-1; c) IL-6; d) IL-8; e) IL-10; and f) neutrophils. 1: nonsmoking controls; 2: smoking controls; 3: mild chronic obstructive pulmonary disease (COPD) patients; 4: moderate COPD patients; 5: severe COPD patient.

controls and to be highest in severe COPD patients (fig. 1). As regards absolute cell counts, there were no differences between smoking controls and COPD patients in regard neutrophils and eosinophils, but a trend to increased macrophage and lymphocyte counts in smoking controls and COPD patients.

#### Cytokine profiles

The mean cytokine concentrations are given in table 3. There was a trend for TNF- $\alpha$  to be higher in smoking controls and COPD patients as compared to controls. Mean IL-6 levels increased virtually linearly from non-smoking controls through smoking controls and mild and moderate COPD to severe COPD. A similar trend was observed for mean IL-8 levels. Conversely, mean IL-1 $\beta$  levels were lowest in moderate and severe COPD patients. IL-10 levels did not reveal any trend (fig. 1).

As regards COPD patients, cytokine levels were not significantly different between current smokers and exsmo-

kers, although there was a trend towards TNF- $\alpha$  IL-8 being higher in current smokers ( $19.1 \pm 12.4$  versus  $10.3 \pm 3.6$ ,  $p=0.055$  and  $571.1 \pm 380.8$  versus  $319.6 \pm 71.2$ ,  $p=0.059$ ).

#### Correlations between forced expiratory volume in one second and cytological cell counts and cytokines levels

FEV1 % pred was significantly inversely correlated with pack-yr of cigarette smoking ( $\rho=-0.47$ ,  $p<0.0001$ ), the percentage of neutrophils ( $\rho=-0.56$ ,  $p<0.0001$ ) IL-6 ( $\rho=-0.37$ ,  $p=0.01$ ) and IL-8 concentration ( $\rho=-0.43$ ,  $p=0.004$ ) in BAL fluid, but not TNF- $\alpha$  (fig. 2).

#### Correlations between percentage of neutrophils in bronchoalveolar lavage fluid and cytokine levels

Significant correlations could be found for the percentage of neutrophils in BAL fluid and pack-yr of cigarette smoking ( $\rho=0.39$ ,  $p=0.01$ ) and IL-8 ( $\rho=0.69$ ,  $p<0.0001$ ), and TNF- $\alpha$  concentration ( $\rho=0.4$ ,  $p<0.005$ ) (fig. 2).

Table 3. – Bronchoalveolar lavage fluid cytokine patterns in chronic obstructive pulmonary disease (COPD) patients and controls

	Control		COPD		
	Nonsmoker	Smoker	Mild	Moderate	Severe
Subjects n	8	9	13	7	10
IL-1 $\beta$ pg·mL <sup>-1</sup>	177 $\pm$ 66.0	205.1 $\pm$ 48.1	208.8 $\pm$ 61.5	105.9 $\pm$ 36.9	127.3 $\pm$ 45.3
IL-6 pg·mL <sup>-1</sup>	3.4 $\pm$ 1.1	8.4 $\pm$ 2.9	17.6 $\pm$ 3.0	18.3 $\pm$ 12.2	40.4 $\pm$ 20.7
IL-8 pg·mL <sup>-1</sup>	48.8 $\pm$ 14.4	287.0 $\pm$ 198.6	255.2 $\pm$ 83.7	292.6 $\pm$ 131.7	673.8 $\pm$ 372.2
IL-10 pg·mL <sup>-1</sup>	10.4 $\pm$ 0.6	8.0 $\pm$ 1.6	14.1 $\pm$ 3.1	11.3 $\pm$ 4.9	9.7 $\pm$ 2.5
TNF- $\alpha$ pg·mL <sup>-1</sup>	7.1 $\pm$ 2.2	12.1 $\pm$ 5.8	9.1 $\pm$ 3.1	17.3 $\pm$ 9.3	15.7 $\pm$ 12.4

Data are presented as mean $\pm$ SD. IL: interleukin; TNF- $\alpha$ : tumour necrosis factor- $\alpha$ . No differences were statistically significant.

### Microbiological results

The PSB technique revealed significant amounts of PPMs in 22 of 64 (34%) patients, and BAL in 14 of 52 (27%) (NS). The kappa coefficient for qualitative agreement on PPMs was 0.39 ( $p=0.002$ ) and for quantitative agreement 0.39 ( $p=0.0009$ ).

Culture results revealed that five of 12 (42%) smoking controls, and nine of 28 (32%) COPD patients with mild, six of 11 (55%) with moderate and two of 13 (15%) with severe airflow limitation had PPMs above the thresholds in PSB samples or BALF (NS for all comparisons). Overall colonization rates were 42% in smoking controls versus 33% in all COPD patients (NS). The corresponding numbers for non-PPMs above the thresholds were seven of 12 (58%) in smoking controls, and 20 of 28 (71%) in COPD patients with mild, three of 11 (27%) with moderate, and five of 13 (39%) with severe airflow limitation. Overall col-

onization rates were 58% in smoking controls versus 54% in all COPD patients (NS).

Table 4 provides an overview of the micro-organisms retrieved. As regards PPMs, the majority were community-acquired pathogens (24 of 27, 89%), whereas only two were Gram-negative enteric bacilli (GNEB) and one *Stenotrophomonas maltophilia*. Colonization patterns were not affected by the severity of airflow obstruction. Within the non-PPM group, *Streptococcus viridans* was the micro-organism most frequently encountered.

### Associations between cytological cell counts, cytokines and microbial patterns

The presence of PPMs in significant amounts was significantly associated with higher neutrophil counts as compared to PPMs in nonsignificant amounts (33.2 $\pm$ 10.4 versus

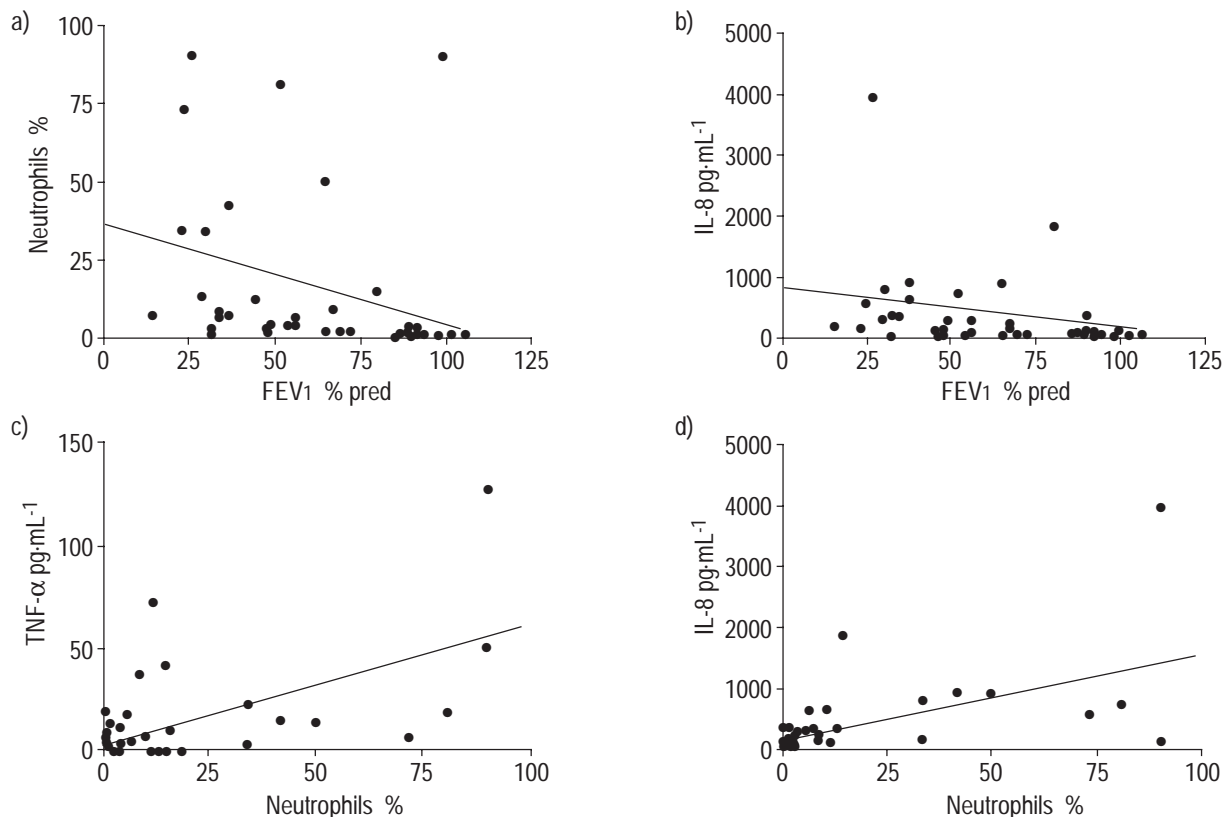


Fig. 2. – Correlations between forced expiratory volume in one second (FEV<sub>1</sub>) and inflammatory markers (a) neutrophils ( $r=-0.56$ ,  $p<0.0001$ ); and b) interleukin-8 (IL-8) ( $r=-0.43$ ,  $p=0.004$ ) and between (c) tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) ( $r=0.4$ ,  $p<0.005$ ); and inflammatory markers and d) IL-8 ( $r=0.69$ ,  $p<0.0001$ ) in bronchoalveolar lavage fluid.

Table 4. – Microbial patterns of controls and chronic obstructive pulmonary disease (COPD) patients

Micro-organism	COPD							
	Control smokers		Mild		Moderate		Severe	
	<CO	≥CO	<CO	≥CO	<CO	≥CO	<CO	≥CO
Subjects n	12		28		11		13	
PPMs								
Community-acquired*	0	6	2	10	0	5	2	3
GNEB+ and <i>P. aeruginosa</i> / <i>S. maltophilia</i>	0	0	1	0	0	2	0	1
Non-PPMs	2	8	8	30	1	4	6	11

\*: *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*; †: *Escherichia coli* and *Proteus mirabilis*. CO: cut-off ( $\geq 10^2$  colony-forming units (cfu)·mL<sup>-1</sup> in protected specimen brush samples and  $\geq 10^3$  cfu·mL<sup>-1</sup> in bronchoalveolar lavage fluid; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. maltophilia*: *Stenotrophomonas maltophilia*.

10.1±3.5%, p=0.02) as well as higher TNF- $\alpha$  levels (29.9±10.8 versus 6.3±2.1 pg·mL<sup>-1</sup>, p=0.01). A nonsignificant trend was also obvious for IL-8 levels (658.9±312.6 versus 311.2±93.9 pg·mL<sup>-1</sup>, p=0.21) (fig. 3).

No corresponding associations could be established for the presence of non-PPMs in significant amounts.

### Discussion

This study yields three main findings. 1) FEV<sub>1</sub> (% pred) was inversely correlated with pack-yr of cigarette smoking and the percentage of neutrophils and IL-6 and IL-8 concentration in BAL fluid. Accordingly, pack-yr of cigarette smoking and IL-8 and TNF- $\alpha$  concentration were positively correlated with the percentage of neutrophils in BAL fluid. 2) Smoking controls and COPD patients were frequently colonized by PPMs in the bronchial tree (33%), mainly by community endogenous pathogens. Colonization rates and patterns of PPMs were not affected by severity of airflow obstruction. 3) However, the presence of significant amounts of PPMs was significantly associated with higher percentages of neutrophils and TNF- $\alpha$  concentration in BAL fluid.

The present study confirms previous findings, from studies using BAL fluid [14, 15] and induced sputum [16], that, in contrast to in asthma, airway inflammation in patients with COPD is characterized mainly by a neutrophilic pattern. However, previous studies using BAL fluid excluded patients with severe COPD for safety reasons [14–16]. These patients were only included in one study

using sputum induction [17]. It was decided to include patients with all degrees of airflow limitation in the present study. A clear trend for the percentage of neutrophils to be higher in smoking controls and mild and moderate COPD patients as compared to nonsmoking controls and to be highest in severe COPD patients was found. Airflow limitation was correlated to pack-yr of cigarette smoking as well as the percentage of neutrophils in BAL fluid, corroborating previous findings of others [14–16]. In accordance with this, increased activity of bronchial neutrophils, as evidenced by increased levels of myeloperoxidase in patients with COPD, has also been described [16, 18].

A corresponding inverse correlation was found with airflow limitation (FEV<sub>1</sub> as % pred) and IL-6 as well as IL-8 concentration. IL-6 is an airway epithelial cell and a macrophage product that exerts pleiotropic pro-inflammatory effects. Thus, it may represent an additional marker of bronchial mucosal inflammation in BAL fluid [19]. Conversely, IL-8 is the main mediator of neutrophil chemotaxis [19]. Similar increasing levels of IL-6 and IL-8 were observed, as with the percentage of neutrophils, dependent on smoking status and the degree of airflow limitation. Increased IL-8 levels in COPD patients were also found by others, using both induced sputum [17] and bronchoalveolar lavage [18]. Conversely, to the authors' knowledge, IL-6 has not been investigated so far in this population. Since IL-8 was correlated with the percentage of neutrophils in BAL fluid, it may mainly reflect neutrophil recruitment [20]. This is also true for TNF- $\alpha$ , which also correlated with the percentage of neutrophils in BAL fluid and which has been shown to increase IL-8 production from

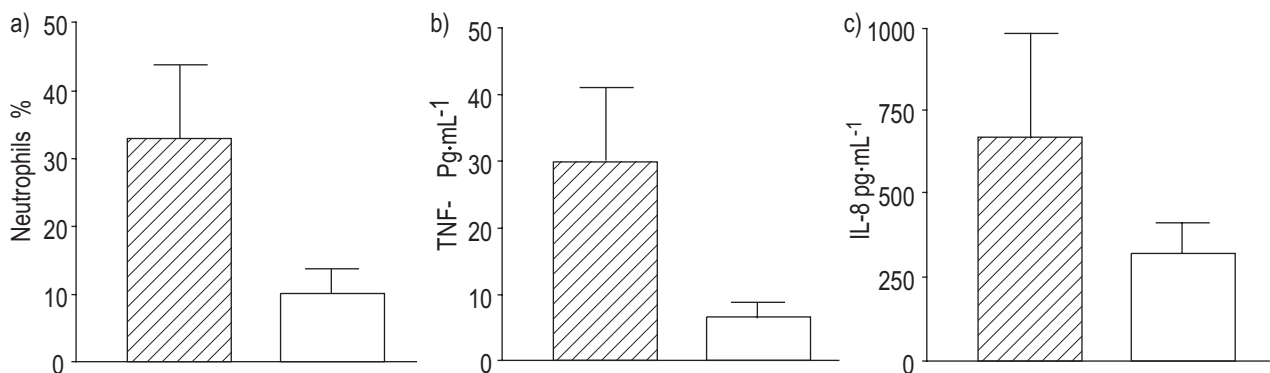


Fig. 3. – Percentage of neutrophils (a, p=0.02) and concentrations of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (b, p=0.01) and interleukin-8 (IL-8) (c, p=0.21) in bronchoalveolar lavage fluid from patients with (▨) and without (□) potentially pathogenic micro-organisms in culture.

epithelial cells and neutrophils [21, 22]. Finally, IL-1 $\beta$  and IL-10 concentrations seemed to be lowest in patients with severe COPD, reflecting upregulation of the inflammatory response [22].

When interpreting colonization rates of COPD patients, it should be realized that these may vary at different times of year or during the course of COPD. Moreover, sampling errors must be taken into account. Nevertheless, keeping in mind these potential limitations, the colonization rates of the bronchial tree obtained were comparable with recent observations of others [4, 23]. PPMs were detected in 42% of smoking controls and 33% of COPD patients, mostly corresponding to community endogenous pathogens. RIISE *et al.* [18] found an overall colonization rate of 37% in smokers with nonobstructive chronic bronchitis as compared to 33% in patients with COPD. The proportions were 11 and 22%, respectively, when only PPMs were taken into account [18]. MONSÓ *et al.* [23] found a colonization rate with PPMs in stable patients with COPD of 25%. A similar low proportion of GNEB and *Pseudomonas* spp. was found (11% in the present study versus 5% in that of MONSÓ *et al.* [23]). As regards non-PPMs, these micro-organisms were found in 58% of smoking controls and 54% of COPD patients, with *S. viridans* the most frequently encountered species. Obviously, smoking and not airflow limitation was the main factor determining airway colonization, corroborating previous findings of others [2]. By the same token, no relationship of the severity of airflow limitation could be demonstrated with either colonization rates or type of PPMs. Likewise, in a recent study of COPD patients with severe exacerbations requiring mechanical ventilation, no association of type of PPMs with severity of airflow limitation could be demonstrated [24]. These observations suggest that there is a general disposition to colonization with PPMs in the presence of airway inflammation.

Conversely, the presence of PPMs was significantly associated with increased percentages of neutrophils and levels of TNF- $\alpha$ . A corresponding trend was obvious for increased IL-8 levels. Others have found that some Gram-positive (e.g. *Streptococcus pneumoniae*) and Gram-negative bacteria stimulate TNF- $\alpha$  production by macrophages [25]. Thus, the present study provides evidence for bronchial colonization with PPMs (and not only infection) being harmful by further enhancing airway inflammation. If these findings could be confirmed in further investigations, there would be a potential role for the prophylactic antimicrobial treatment of patients with COPD and proven bronchial colonization with PPMs. Corresponding reductions in IL-1 and TNF- $\alpha$  concentration in the sputum of patients with bronchiectasis after a clinical response to antimicrobial treatment have been recently demonstrated [26]. Conversely, there was no evidence for a comparable role of non-PPMs as promoters of bronchial inflammation.

A general methodological problem of BAL studies is the lack of an accepted reference to compensate for the dilution of lavaged fluid. In the present study, decreasing recovery of BAL fluid with increasing severity of COPD might have influenced the results of cytokine measurements. However, it seems that a decreased recovery should lead to an underestimation of cytokine concentrations, since endothelial lining fluid sampling might have been rendered incomplete. Therefore, it is thought that differences in recovery are not likely to result in type-1 errors as regards the

association of cytokine levels with pulmonary function and colonization. Another concern may be that cytokine levels in COPD patients may have been affected by current smoking. However, the differences in cytokine level were nonsignificant. For this reason, the increased TNF- $\alpha$  and IL-8 levels in current smokers might have impeded even more striking differences between COPD groups. Finally, inhaled steroid treatment may influence the cytological and cytokine patterns. However, there were no significant differences in any parameter when calculations were repeated in subgroups receiving or not receiving inhaled steroids (data not shown). Instead, there was a uniform trend towards higher percentages of neutrophils and levels of cytokines in COPD patients inhaling steroids, indicating that inhaled steroids were prescribed to patients with higher inflammatory activity. Thus, it cannot be excluded that differences in COPD patients as compared to controls would have been more pronounced in the absence of inhaled steroid treatment.

In conclusion, the present study confirms bronchial neutrophilia as a key inflammatory pattern in chronic obstructive pulmonary disease patients which may predispose to bronchial bacterial colonization. Moreover, it provides evidence that bronchial colonization with potentially pathogenic micro-organisms represents an independent stimulus for additional airway inflammation.

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