Biological Markers in Induced Sputum of Patients with Different Phenotypes of Chronic Airway Obstruction

Maria Laura Bartoli Antonella Di Franco Barbara Vagaggini Elena Bacci
Silvana Cianchetti Federico Lorenzo Dente Monica Tonelli Pier Luigi Paggiaro

Cardiothoracic and Vascular Department, University of Pisa, Pisa, Italy

Key Words

Chronic obstructive pulmonary disease • Induced sputum • Chronic asthma • Chronic bronchitis • Emphysema

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease characterized by a combination of 3 different disorders, namely chronic asthma, chronic bronchitis and pulmonary emphysema, sometimes simultaneously present in the same subject. **Objectives:** The aim of our study was to compare sputum inflammatory markers in patients with different phenotypes of chronic airway obstruction. **Methods:** Forty-five subjects (forced expiratory volume in 1 s/vital capacity, FEV₁/VC: 58.8 ± 12.2%; FEV₁: 49.8 ± 11.5% of predicted) were classified as chronic asthma (n = 10) or COPD patients (n = 35); the latter were further divided into patients with prevalent chronic bronchitis (n = 24) or prevalent pulmonary emphysema (n = 11) according to clinical history and functional evaluation, and underwent sputum induction and analysis of inflammatory cell and soluble mediators. **Results:** Patients with chronic asthma showed higher sputum eosinophil percentages and eosinophilic cationic protein levels, and lower neutrophil percentages and neutrophil elastase levels than COPD patients. Neutrophil chemotactic activity in sputum supernatant was higher than the pool of normal subjects both in chronic asthma and COPD patients. No difference in sputum cell compo-

sition and levels of soluble mediators was observed between patients with chronic bronchitis and patients with pulmonary emphysema. **Conclusions:** The pattern of airway inflammation in induced sputum of patients with chronic asthma is different from that of COPD patients with a similar FEV₁. Among COPD patients, however, the pattern of airway inflammation shows no difference between chronic bronchitis and patients with pulmonary emphysema, suggesting that these two clinically and functionally distinct phenotypes share a common inflammatory pattern as detected by induced sputum.

Copyright © 2008 S. Karger AG, Basel

Introduction

Chronic airway obstruction is one of the most common functional abnormalities in European countries [1], characterized by persistent airflow limitation, irreversible even after treatment. This syndrome is often due to a mixture of three distinct diseases: chronic asthma, chronic bronchitis and pulmonary emphysema, which sometimes may be simultaneously present in the same subject, each to a different degree [2]. All these conditions have an inflammatory background, with the involvement of several cells and mediators [3, 4]. While asthma can be easily differentiated from the other two diseases on the basis of clinical and functional findings, chronic bron-

chitis and emphysema, despite having typically distinct features, are often gathered under the common term of 'chronic obstructive pulmonary disease' (COPD) [2].

The heterogeneity of chronic airway obstruction is important in terms of pathophysiology and prognosis. While chronic asthma is characterized by a distinct airway inflammatory pattern [5–7] and a lower mortality rate [8] than COPD with a similar degree of airway obstruction, chronic bronchitis and pulmonary emphysema, despite having different pathophysiological features, have not been clearly differentiated in terms of biological markers, response to treatment and prognosis. Patients with emphysema have slightly higher levels of sputum CD4 lymphocytes [9], higher percentages of sputum eosinophils and a higher matrix metalloproteinase 9/tissue inhibitor of metalloproteinase 1 ratio in sputum supernatant [10]. Papi et al. [11] reported that COPD patients with reversible acute airway obstruction have higher levels of exhaled nitric oxide than patients with irreversible COPD, but no further distinction was made between chronic bronchitis and pulmonary emphysema.

The aim of this study was to evaluate whether sputum inflammatory markers distinguish different phenotypes of chronic airway obstruction (chronic asthma vs. chronic bronchitis vs. pulmonary emphysema). This difference might be useful for a better definition of these different diseases, and possibly for a more accurate therapeutic approach.

Patients and Methods

Subjects

We studied 45 patients (35 males) with chronic airway obstruction who had been regularly followed at our clinic for at least 2 years. They were recruited consecutively from our clinic where they had attended as outpatients for 12 months. The main inclusion criterion was the persistence of airway obstruction after bronchodilation [forced expiratory volume in 1 s/vital capacity (FEV₁/VC) <88–89% of predicted, and FEV₁% <70% of predicted], documented several times during the whole observation period as outpatients in our clinic. Subjects with bronchiectasis, previous tuberculosis, and/or known respiratory diseases other than asthma and COPD were excluded. All patients were examined in a stable phase of the disease, at least 8 weeks after the last exacerbation.

The mean age of patients was 68.5 ± 9.3 years, 39 were smokers or ex-smokers, 9 were atopic, as assessed by skin prick tests to common airborne allergens. Mean duration of the disease was 17.9 ± 11 years. At the time of enrolment, postbronchodilation FEV₁% was 49.7 ± 11.5 and FEV₁/VC% was 58.8 ± 12.2 of predicted values. All patients were regularly treated with moderate to high doses of inhaled corticosteroids (ICS: 1,000–2,000 µg daily of beclomethasone dipropionate or equivalent) and with long-

acting β_2 -agonists (salmeterol in 32 and formoterol in 13 patients); in 24 out of 35 COPD patients, tiotropium was also used, while slow-release oral theophylline was taken by 12 out of 45 patients. No patients were under regular oral corticosteroids.

All patients were examined on two different days. On the first day, the selection criteria were confirmed, and complete pulmonary function tests, including the measurement of total lung capacity (TLC), single-breath diffusing capacity of the lung for carbon monoxide (DLCO_{sb}) and DLCO/alveolar volume (VA) (Medical Graphics, Pulmonary System, Milan, Italy), were performed; reversibility after 400 µg salbutamol inhalation was also measured. Arterial blood was sampled at rest for gas analysis. On the second day, 3 days after withdrawal of regular pharmacological treatment, induced sputum was collected after salbutamol pretreatment.

The study was approved by the Institutional Review Board, and each patient gave informed consent.

Definition of Phenotypes

Patients were divided into asthmatic and COPD patients according to the following criteria: (a) asthma – patients with a long history of attacks of shortness of breath with wheezing starting before the age of 40, with no relevant smoking history (nonsmokers, ex-smokers or current smokers smoking less than 10 pack-years); (b) COPD – history of chronic cough and/or dyspnea, beginning after the age of 40, along with smoking habit (more than 10 pack-years).

COPD patients were further divided into prevalent chronic bronchitis and prevalent pulmonary emphysema according to the following criteria: (a) chronic bronchitis – patients with clinical history of chronic cough and phlegm and increased bronchial markings on chest X-ray, regardless of TLC and DLCO/VA values; (b) pulmonary emphysema – patients with dyspnea on exercise (at least score 2 of Medical Research Council dyspnea scale) associated with TLC >120% of predicted value and DLCO/VA <60% of predicted value, with or without clear findings of pulmonary hyperinflation on chest X-ray.

Sputum Induction and Analysis

Sputum induction and analysis were carried out according to a previously published standardized protocol [12]. After inhaled salbutamol pretreatment, saline solution was delivered by means of an ultrasonic nebulizer (2.8 ml/min output; Sirius, Technomed, Florence, Italy). In 39 patients, hypertonic solution (NaCl 4.5% w/v) was used to induce sputum production; in the remaining 6 patients, all with FEV₁ <35%, normal saline (NaCl 0.9% w/v) was used, as recommended [12]. Nebulization was stopped after 15 min or earlier if FEV₁ fell by 20% or more from baseline values. Before and every 5 min after the start of nebulization, FEV₁ was measured and patients were asked to rinse their mouths and throats carefully to discard saliva and to try to cough sputum into a container.

Sputum samples were processed within 2 h from collection, and more viscid and denser portions were selected and processed as previously described [13]. Briefly, samples were homogenized by adding 0.1% dithiothreitol in a shaking bath at 37°C for 15 min and centrifuged to separate cells from supernatant. Supernatant was stored at –80°C for further analysis. The cell pellet was resuspended in phosphate-buffered saline for viability test and total cell count; aliquots were used to prepare slides for differential cell counts. At least 300 inflammatory cells were counted. Macro-

phage, lymphocyte, neutrophil, eosinophil values were expressed as percent of total inflammatory cells.

Slides with cell viability <50% and an amount of squamous cells such that 300 inflammatory cells could not be counted were considered inadequate and discarded. Reproducibility for sputum inflammatory cell count had been previously assessed on a different group of 20 patients, and it was satisfactory, excluding lymphocytes (RI: 0.23): RI was 0.8 for macrophages, 0.85 for neutrophils, 0.87 for eosinophils [14].

Cytokines in Sputum Supernatant

Sputum interleukin (IL)-8 levels were measured by means of a commercially available enzyme immunoassay (Euroclone, Milan, Italy) according to the manufacturer's recommended protocol. The assay detection limit was 15 pg/ml. The percentage of recovery, evaluated in 10 different samples by the spiking method, was 89.4%, in the presence of 0.1% dithiothreitol. IL-8 intra- and interassay coefficients of variation were 4.7 and 7.5%, respectively [13].

Neutrophilic/Eosinophilic Biomarkers in Sputum Supernatant

Sputum eosinophil cationic protein (ECP) levels were measured according to the manufacturer's recommended protocol by means of a commercially available fluoroimmunoassay (Pharmacia, Uppsala, Sweden). The assay detection limit was 0.5 µg/l. The intra- and interassay coefficients of variation were 4.5 and 8.2%, respectively [13].

Sputum neutrophilic elastase activity (NE) was measured spectrophotometrically using the synthetic substrate methoxysuccinyl-ala-ala-pro-val-paranitroanilide (Sigma-Aldrich Co. Ltd., Poole, UK) [13]. The activity was assessed by measuring the absorbance at 410 nm on a microplate reader, and quantified by extrapolation on a standard curve of pure NE. The assay detection limit was 0.006 µg/ml. Mean intra- and interassay coefficients of variation were 5.6 and 12.0%, respectively.

Neutrophil Chemotactic Activity

Neutrophil chemotactic activity (NCA) in sputum supernatant was measured by means of a 48-well microchemotaxis Boyden chamber (Neuroprobe; Cabin John, Md., USA). Sputum supernatants (27 µl) at different dilutions (1:2, 1:5, 1:10, 1:20) or controls, in triplicate, were placed in the lower wells of the chamber and used as chemoattractant. Nitrocellulose filters (130 µm thickness and 5 µm pore size, Neuroprobe) were placed over the samples. A suspension of healthy donor neutrophils (50 µl) was added in the upper wells of the chamber and placed on top of the filter. The chamber was then incubated at 37°C and 5% CO₂ for 45 min. Neutrophil migration was evaluated by means of the 'leading front technique' and expressed as distance (µm) run by neutrophils through the filter, or as percent migration increase in comparison with the migration induced by a pool of sputum supernatants obtained from normal patients. Mean NCA intra- and interassay coefficients of variation were 4.8 and 12.3%, respectively.

Statistical Analysis

Pulmonary function parameters are expressed as means ± SD. Sputum cell viability, total cell counts, cell percentages, and biochemical mediators are expressed as medians and ranges.

Differences between two groups (asthma vs. COPD, or chronic bronchitis vs. emphysema) were tested by means of the t test for

Table 1. Characteristics of the examined subjects

a Characteristics of patients with chronic asthma and COPD

	Chronic asthma	COPD
Number	10	35
Age, years	58.0 ± 10.5	71.9 ± 5.7 ^a
Sex (M/F)	6/4	29/6
Atopy (yes/no)	7/3	2/33
Smoking habit (yes/no/ex)	1/6/3	3/0/32
Pack-years	5.2 ± 2.1 ¹	39.0 ± 23.4 ^a
Disease duration, years	26.4 ± 10.8	15.0 ± 9.1 ^a

b Characteristics of patients with chronic bronchitis and emphysema

	Chronic bronchitis	Emphysema
Number	24	11
Age, years	73.7 ± 4.3	68.8 ± 6.7 ^b
Sex (M/F)	22/2	7/4 ^b
Atopy (yes/no)	1/23	1/10
Smoking habit (yes/no/ex)	2/0/22	1/0/10
Pack-years	41.6 ± 25.6	34.5 ± 18.9
Disease duration, years	15.9 ± 10.4	13.5 ± 6.4

^a p < 0.05 between chronic asthma and COPD; ^b p < 0.05 between chronic bronchitis and emphysema.

¹ In 4 smokers or ex-smokers.

normally distributed variables and the Mann-Whitney test for nonnormally distributed variables. Differences among three groups (asthma vs. chronic bronchitis vs. emphysema) were tested by the ANOVA for normally distributed variables and the Kruskal-Wallis test for nonnormally distributed variables.

Correlations between functional and biological parameters, and inflammatory cells and biochemical mediators were evaluated by means of Spearman's rank correlation test. p < 0.05 was considered significant.

Results

As expected, chronic asthmatics were younger, had a longer history of disease and smoked less than COPD patients (table 1). No difference in smoking habit and disease duration was found between patients with chronic bronchitis and patients with pulmonary emphysema, but the female gender was more represented in the emphysema group, and the mean age of patients with prevalent emphysema was lower than in patients with prevalent chronic bronchitis.

Table 2. Functional parameters of the examined subjects**a** Functional parameters of patients with chronic asthma and COPD

	Chronic asthma	COPD
FEV ₁ , % predicted	46.2 ± 6.9	50.9 ± 12.6
FEV ₁ /VC, %	54.8 ± 9.2	60.2 ± 12.8
Δ FEV ₁ after bronchitis, %	14.8 ± 9.7	7.6 ± 9.3*
VC, % predicted	88.6 ± 19.4	86.5 ± 18.7
TLC, % predicted	119.4 ± 12.3	114.4 ± 25.8
DLCO _{sb} , % predicted	85.7 ± 23.7	63.9 ± 18.7*
DLCO/VA	92.8 ± 19.8	70.1 ± 18.9*
PO ₂ , mm Hg	79.2 ± 11.3	73.7 ± 9.3
PCO ₂ , mm Hg	38.4 ± 4.2	40.2 ± 5.0

b Functional parameters of patients with chronic bronchitis and emphysema

	Chronic bronchitis	Emphysema
FEV ₁ , % predicted	50.7 ± 12.4	51.3 ± 13.4
FEV ₁ /VC, %	64.6 ± 12.6	52.5 ± 9.7**
Δ FEV ₁ after bronchitis, %	8.3 ± 10.6	6.3 ± 6.3
VC, % predicted	80.2 ± 13.9	97.5 ± 21.5**
TLC, % predicted	105.8 ± 25.5	129.4 ± 18.9**
DLCO _{sb} , % predicted	69.0 ± 16.6	54.9 ± 19.5
DLCO/VA	77.4 ± 17.2	57.3 ± 14.9**
PO ₂ , mm Hg	73.1 ± 8.1	74.7 ± 11.4
PCO ₂ , mm Hg	40.4 ± 4.0	39.7 ± 6.6

* p < 0.05 between chronic asthma and COPD; ** p < 0.05 between chronic bronchitis and emphysema.

Table 3. Biological markers measured in induced sputum of subjects with chronic asthma or COPD

	Chronic asthma	COPD
Number	10	35
Inflammatory cells/ml·10 ⁶	2.2 (0.7–10.0)	3.3 (0.4–23.0)
Macrophages, cells/ml·10 ⁶	0.4 (0.2–7.4)	0.6 (0.05–6.2)
Lymphocytes, cells/ml·10 ⁶	0.03 (0–0.2)	0.03 (0–0.2)
Neutrophils, cells/ml·10 ⁶	1.0 (0.2–4.9)	2.6 (0.05–17.3)*
Eosinophils, cells/ml·10 ⁶	0.1 (0.01–1.9)	0.05 (0–0.7)*
Macrophages, %	24.0 (6.7–76.7)	17.8 (2.0–85.5)
Lymphocytes, %	0.6 (0–4.3)	0.9 (0–3.6)
Neutrophils, %	52.6 (6.6–83.9)	76.8 (12.0–96.6)*
Eosinophils, %	6.5 (0.3–83.5)	1.5 (0–33.3)*
ECP, µg/ml	8.6 (0.5–19.5)	1.0 (0–17.1)*
IL-8, ng/ml	15.7 (1.3–278.4)	23.8 (2.6–99.0)
Elastase, µg/ml	0.02 (0–11.4)	6.8 (0–14.6)*

* p < 0.05 between asthma and COPD.

Table 4. Biological markers measured in induced sputum of subjects with prevalent chronic bronchitis or with prevalent emphysema

	Chronic bronchitis	Emphysema
Number	24	11
Inflammatory cells/ml·10 ⁶	2.8 (0.4–13.0)	5.2 (0.8–23.0)
Macrophages, cells/ml·10 ⁶	0.6 (0.1–2.8)	0.4 (0.1–6.2)
Lymphocytes, cells/ml·10 ⁶	0.03 (0–0.1)	0.02 (0–0.2)
Neutrophils, cells/ml·10 ⁶	1.8 (0.5–10.0)	4.9 (0.4–17.3)*
Eosinophils, cells/ml·10 ⁶	0.05 (0–0.7)	0.05 (0–0.5)
Macrophages, %	22.2 (4.9–85.5)	14.2 (2.0–33.6)
Lymphocytes, %	1.1 (0–3.6)	0.6 (0–1.0)
Neutrophils, %	71.9 (12.0–93.2)	84.1 (47.8–96.6)*
Eosinophils, %	1.6 (0.0–33.3)	1.2 (0–23.3)
ECP, µg/ml	1.1 (0–17.1)	0.5 (0.0–1.8)
IL-8, ng/ml	18.4 (2.8–78.9)	31.5 (2.5–99.0)
Elastase, µg/ml	9.5 (0.0–14.6)	6.7 (3.5–10.8)

* p = 0.057 in comparison with chronic bronchitis.

Patients with chronic asthma had significantly higher DLCO_{sb}% and DLCO/VA values than COPD patients (table 2). Among COPD patients, those with chronic bronchitis had lower VC% and TLC% values and a higher FEV₁/VC% ratio than patients with pulmonary emphysema.

Patients with chronic asthma had higher sputum levels of eosinophils (cells/ml and percent) and of ECP, and lower levels of neutrophils (cells/ml and percent) and NE than patients with COPD (table 3). Among COPD patients, no difference in sputum inflammatory cells and mediators was found between chronic bronchitis and emphysema (table 4), although neutrophils (cells/ml and percent) tended to be higher in patients with prevalent emphysema than in patients with prevalent chronic bronchitis. Sputum cell and mediator findings of the three different phenotypes (chronic asthma, chronic bronchitis and emphysema) are reported in figure 1.

Except for 2 patients, all samples showed NCA values higher than those measured in a pool of sputum supernatant obtained from a group of control patients. No difference was found in absolute migration (micrometers) at different dilutions or in percent migration increase between different groups of study patients (table 5).

Considering all the patients together, a negative correlation was found between FEV₁% and the percentage of neutrophils in induced sputum (p < 0.02, ρ = -0.46). As

Fig. 1. Eosinophil and neutrophil percentages in induced sputum, and sputum supernatant ECP and NE concentrations in the three different phenotypes of chronic airway obstruction. ^a $p < 0.05$; ^b $p = 0.06$.

expected, there was a positive correlation between sputum eosinophils (percent and cells/ml) and sputum ECP ($p = 0.008$, $\rho = 0.5$ and $p = 0.001$, $\rho = 0.6$, respectively), and between sputum neutrophils (percent and cells/ml) and sputum NE ($p = 0.04$, $\rho = 0.34$ and $p = 0.04$, $\rho = 0.33$, respectively).

The comparison between COPD patients with different acute reversibility to inhaled salbutamol did not show significant differences for any of the inflammatory markers measured in sputum (data are reported in the appendix).

Discussion

The aim of this study was to evaluate whether sputum inflammatory markers distinguish the different phenotypes of chronic airway obstruction. At present, no gold standard is available to discriminate chronic asthma, chronic bronchitis and pulmonary emphysema from each other, but the combination of different clinical and functional findings allows a reasonably accurate identification of the different disease subgroups. Our data con-

firm previous observations [5–7] showing that airway inflammation in chronic asthma is different from that observed in chronic bronchitis and emphysema, with eosinophils and their mediators being higher in airway secretions of chronic asthmatics, and neutrophils and their mediators (in particular NE), being higher in chronic bronchitis and emphysema. On the other hand, we found no significant difference in sputum markers between patients with prevalent chronic bronchitis and patients with prevalent emphysema, suggesting that such a clinical and functional heterogeneity may not reflect a similar heterogeneity in the underlying inflammatory pattern.

The cytokine pattern observed in sputum supernatant or in bronchoalveolar lavage fluid is different in asthmatic and COPD patients [15, 16], even if the level of airway obstruction is similar [5], and airway biopsies also show important differences between asthmatic and COPD patients [16]. Our patient selection was different from that reported by many published papers [6, 15, 16], which compared young asthmatics with normal or nearly normal pulmonary function to older COPD patients with moderate to severe airway obstruction. Only Fabbri

Table 5. NCA¹ and Δ NCA² measured in induced-sputum supernatant obtained from the three different phenotypes of chronic airway obstruction

a NCA and Δ NCA measured in induced-sputum supernatant obtained from patients with chronic asthma and COPD

	Chronic asthma	COPD
NCA, μ m		
Dilution 1:2	104.1 $\pm$ 8.5	101.3 $\pm$ 12.2
Dilution 1:5	88.9 $\pm$ 8.2	92.9 $\pm$ 9.9
Dilution 1:10	79.5 $\pm$ 6.4	85.8 $\pm$ 10.6
Dilution 1:20	77.6 $\pm$ 6.4	81.6 $\pm$ 10.4
Δ NCA, %		
Dilution 1:2	24.3 $\pm$ 20.1	20.6 $\pm$ 15.4
Dilution 1:5	19.3 $\pm$ 18.5	20.3 $\pm$ 10.3
Dilution 1:10	13.1 $\pm$ 12.9	19.9 $\pm$ 14.8
Dilution 1:20	6.8 $\pm$ 9.1	18.1 $\pm$ 16.1

b NCA and Δ NCA measured in induced-sputum supernatant obtained from patients with chronic bronchitis and emphysema

	Chronic bronchitis	Emphysema
NCA, μ m		
Dilution 1:2	101.2 $\pm$ 13.3	101.6 $\pm$ 10.5
Dilution 1:5	93.7 $\pm$ 10.9	91.5 $\pm$ 7.9
Dilution 1:10	86.2 $\pm$ 11.1	85.0 $\pm$ 10.1
Dilution 1:20	82.7 $\pm$ 11.3	79.8 $\pm$ 9.0
Δ NCA, %		
Dilution 1:2	20.5 $\pm$ 18.3	20.9 $\pm$ 9.7
Dilution 1:5	22.1 $\pm$ 10.8	16.9 $\pm$ 8.9
Dilution 1:10	23.8 $\pm$ 13.2	18.4 $\pm$ 17.5
Dilution 1:20	20.0 $\pm$ 16.4	15.0 $\pm$ 16.0

¹ Migration (mean $\pm$ SD, μ m) was measured at different dilutions.

² Δ NCA (mean $\pm$ SD, %) compared with migration induced by a control pool at the same dilution.

et al. [5] compared asthmatic and COPD patients of similar ages and levels of airway obstruction, but only sputum cell counts were reported. We extended the results of this and previous papers and showed that markers of neutrophil and eosinophil activation (NE and ECP) are increased to a different extent as well in sputum supernatant of asthmatic and COPD patients. Furthermore, these markers were significantly higher in our patients than in a group of normal subjects previously studied according to a similar protocol [17]. In contrast to other authors who examined young patients with mild to mod-

erate asthma, we did not observe any significant difference in sputum IL-8 concentrations [6]. It is well known that sputum IL-8 concentrations and neutrophil counts are related to the level of chronic airway obstruction, which is frequently related to the duration of asthma [18]. Furthermore, we have demonstrated higher levels of sputum IL-8 concentrations in patients with severe asthma with persistent airway obstruction than in patients with severe asthma but normal pulmonary function [17]. Therefore, sputum IL-8 concentrations appear to be related to the chronicity of the disease both in asthma and in COPD.

Increased sputum eosinophils have been found in patients with COPD, both in stable phases [19] and during acute exacerbation [20], but their percentages were always lower than those observed in asthmatic patients [5–7]. In our study, 6 out of 10 asthmatic patients and 9 out of 35 COPD patients, without a difference between COPD phenotypes, had sputum eosinophil percentages greater than 3% (the upper limit of normal distribution in a large sample of healthy patients in Italy) [21]. Some authors have reported sputum eosinophilia in patients with pulmonary emphysema [22] without comparing them with other COPD phenotypes, however. The meaning of sputum eosinophilia in COPD patients is not known, but it has been associated with a better response to corticosteroids [23].

In all the examined patients, sputum NCA was higher than NCA measured in a pool of sputum samples obtained from normal patients. Other authors found high NCA in sputum supernatant of COPD patients, largely due to the presence of IL-8 and leukotriene B₄ [24]. We found no difference in NCA mean values between patients with chronic asthma and patients with COPD, suggesting that chemotactic factors for neutrophils are equally released in the airways of asthmatic and COPD patients. This observation is in agreement with the similar levels of neutrophils and IL-8 in asthmatic and COPD patients.

We found no relevant difference in sputum inflammatory markers between patients with prevalent chronic bronchitis and patients with prevalent pulmonary emphysema. Very few studies have addressed this issue [9, 10], showing only negligible differences in sputum lymphocyte subpopulations. The distinction between chronic bronchitis and emphysema is important: in some cases, clinical and functional findings allow this distinction, but in many cases the two phenotypes overlap, and no single measurement can be considered the 'gold standard' to distinguish one phenotype from the other, al-

though the measurement of areas with low attenuation clusters on chest CT scans might play a relevant role [25]. We distinguished pulmonary emphysema from chronic bronchitis mainly on the basis of objective evaluations, according to previous papers showing that TLC, and even more DLCO, had the highest correlation coefficients with CT emphysema score [26].

We found no difference between patients with reversible and irreversible COPD as regards sputum inflammatory markers. Some previous studies reported that patients with reversible COPD had higher levels of exhaled nitric oxide [11], blood or sputum eosinophils [11, 27] or higher leukotriene B₄ concentrations in breath condensate or sputum supernatant [28]. However, the relevance of a single acute bronchodilator response in COPD patients is questionable because acute reversibility may change over time in the same COPD patients [29]. Indeed, acute reversibility is no longer considered an exclusion criterion for the diagnosis of COPD [30].

Our study has some limitations. Although patients with known bronchiectasis were not considered for this study, we cannot exclude that patients with chronic asthma or COPD may have mild clinically asymptomatic bronchiectasis; however, in a minority of patients, high-resolution CTs were available, and no bronchiectasis was detected. Secondly, the presence of bacterial colonization was not assessed in all subjects, although only a minority of subjects who underwent a bacterial examination of the sputum showed the presence of pathogenic bacteria in the stable phase of the disease. Furthermore, induced sputum samples can be derived from proximal airways, and in chronic bronchitis and emphysema, pathologic findings can be located in different airway generations (proximal vs. distal airways). All our patients were regularly treated with moderate to high doses of ICS; although they had withdrawn all treatments 3 days before sputum measurement, the efficacy of ICS on sputum measurements in COPD is controversial [31–34]. Furthermore, 4 out of 45 patients were current smokers, while 3 other asthmatic subjects were ex-smokers, and this may have influenced the sputum characteristics [35]. Therefore, we cannot exclude that these factors may have modified the characteristics of airway inflammation in our patients.

In conclusion, we confirm that the pattern of airway inflammation in induced sputum of patients with chronic asthma is different from that of COPD patients with similar FEV₁ levels. By contrast, we found no significant difference between patients with prevalent chronic bronchitis and patients with prevalent pulmonary emphysema,

suggesting that these two clinical and functional phenotypes may have a similar inflammatory pattern as detected by induced sputum.

Acknowledgements

We thank Ms. Mariella De Santis and Ms. Elisa Masino for performing the functional evaluations and sputum induction. This work was partially supported by an educational grant of Boehringer Ingelheim, Italy.

Appendix

Biological parameters of COPD subjects according to reversibility of airway obstruction after inhaled salbutamol.

	Subjects with reversible airway obstruction	Subjects with irreversible airway obstruction
Number	11	24
Inflammatory cells/ml·10 ⁶	2.8 (0.5–8.2)	3.4 (0.4–23.0)
Macrophages, cells/ml·10 ⁶	0.6 (0.07–2.8)	0.6 (0.05–6.2)
Lymphocytes, cells/ml·10 ⁶	0.01 (0–0.1)	0.03 (0–0.2)
Neutrophils, cells/ml·10 ⁶	1.7 (0.3–5.4)	2.8 (0.05–17.3)
Eosinophils, cells/ml·10 ⁶	0.07 (0–0.7)	0.03 (0–0.5)
Macrophages, %	19.5 (5.4–55.6)	17.8 (2.0–85.5)
Lymphocytes, %	0.7 (0–3.6)	0.9 (0–3.4)
Neutrophils, %	74.0 (28.7–94.6)	76.8 (12.0–96.6)
Eosinophils, %	1.9 (0–33.3)	1.3 (0–23.3)
ECP, µg/ml	0.4 (0.04–9.1)	1.0 (0–17.0)
IL-8, ng/ml	22.1 (2.5–63.5)	26.0 (2.8–99.0)
Elastase, µg/ml	7.2 (0–13.6)	6.8 (0–14.6)

References

- 1 Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, Mannino DM: Global burden of COPD: systematic review and meta-analysis. *Eur Respir J* 2006;28:523–532.
- 2 American Thoracic Society: Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:s77–s121.
- 3 Chung KF, Adcock IM: Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J* 2008;31:1334–1356.
- 4 Holgate ST: Pathogenesis of asthma. *Clin Exp Allergy* 2008;38:872–897.
- 5 Fabbri LM, Romagnoli M, Corbetta L, Casoni G, Busljetic K, Turato G, Ligabue G, Ciaccia A, Saetta M, Papi A: Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003;167:418–424.

- 6 Keatings VM, Collins PD, Scott DM, Barnes PJ: Differences in interleukin-8 and tumor necrosis factor- α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530–534.
- 7 Keatings VM, Barnes PJ: Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997;155:449–453.
- 8 Burrows B, Bloom JW, Traver GA, Cline MG: The course and prognosis of different forms of chronic airways obstruction in a sample from the general population. *N Engl J Med* 1987;317:1309–1314.
- 9 Boschetto P, Miniati M, Miotto D, Braccioni F, De Rosa E, Bononi I, Papi A, Saetta M, Fabbri LM, Mapp CE: Predominant emphysema phenotype in chronic obstructive pulmonary disease patients. *Eur Respir J* 2003;21:450–454.
- 10 Boschetto P, Quintavalle S, Zeni E, Leprotti S, Potena A, Ballerin L, Papi A, Palladini G, Luisetti M, Annovazzi L, Iadarola P, De Rosa E, Fabbri LM, Mapp CE: Association between markers of emphysema and more severe chronic obstructive pulmonary disease. *Thorax* 2006;61:1037–1042.
- 11 Papi A, Romagnoli M, Baraldo S, Braccioni F, Guzzinati I, Saetta M, Ciaccia A, Fabbri LM: Partial reversibility of airflow limitation and increased exhaled NO and sputum eosinophilia in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;162:1773–1777.
- 12 Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanović R, Maestrelli P, Sterk PJ: Sputum induction. *Eur Respir J Suppl* 2002;37:3s–8s.
- 13 Cianchetti S, Bacci E, Bartoli ML, Ruocco L, Pavia T, Dente FL, Di Franco A, Vagaggini B, Paggiaro P: Can hypertonic saline inhalation influence preformed chemokine and mediator release in induced sputum of chronic obstructive pulmonary disease patients? Comparison with isotonic saline. *Clin Exp Allergy* 2007;37:1819–1826.
- 14 Bacci E, Cianchetti S, Carnevali S, Bartoli ML, Dente FL, Di Franco A, Giannini D, Vagaggini B, Paggiaro PL: Induced sputum is a reproducible method to assess airway inflammation in asthma. *Mediators Inflamm* 2002;11:293–298.
- 15 Lacoste JY, Bousquet J, Chanez P, Van Vyve T, Simony-Lafontaine J, Lequeu N, Vic P, Enander I, Godard P, Michel FB: Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 1993;92:537–548.
- 16 Leckie MJ, Jenkins GR, Khan J, Smith SJ, Walker C, Barnes PJ, Hansel TT: Sputum T lymphocytes in asthma, COPD and healthy subjects have the phenotype of activated intraepithelial T cells (CD69+ CD103+). *Thorax* 2003;58:23–29.
- 17 Dente FL, Carnevali S, Bartoli ML, Cianchetti S, Bacci E, Di Franco A, Vagaggini B, Paggiaro P: Profiles of proinflammatory cytokines in sputum from different groups of severe asthmatic patients. *Ann Allergy Asthma Immunol* 2006;97:312–320.
- 18 Little SA, MacLeod KJ, Chalmers GW, Love JG, McSharry C, Thomson NC: Association of forced expiratory volume with disease duration and sputum neutrophils in chronic asthma. *Am J Med* 2002;112:446–452.
- 19 Balzano G, Stefanelli F, Iorio C, De Felice A, Melillo EM, Martucci M, Melillo GL: Eosinophilic inflammation in stable chronic obstructive pulmonary disease. Relationship with neutrophils and airway function. *Am J Respir Crit Care Med* 1999;160:1486–1492.
- 20 Zhu J, Qiu YS, Majumdar S, Gamble E, Martin D, Turato G, Fabbri LM, Barnes N, Saetta M, Jeffery PK: Exacerbations of bronchitis: bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants. *Am J Respir Crit Care Med* 2001;164:109–116.
- 21 Spanevello A, Confalonieri M, Sulotto F, Romano F, Balzano G, Migliori GB, Bianchi A, Michetti G: Induced sputum cellularity. Reference values and distribution in normal volunteers. *Am J Respir Crit Care Med* 2000;162:1172–1174.
- 22 Fujimoto K, Kubo K, Yamamoto H, Yamaguchi S, Matsuzawa Y: Eosinophilic inflammation in the airway is related to glucocorticoid reversibility in patients with pulmonary emphysema. *Chest* 1999;115:697–702.
- 23 Chanez P, Vignola AM, O'Shaughnessy T, Enander I, Li D, Jeffery PK, Bousquet J: Corticosteroid reversibility in COPD is related to features of asthma. *Am J Respir Crit Care Med* 1997;155:1529–1534.
- 24 Beeh KM, Kornmann O, Buhl R, Culpitt SV, Giembycz MA, Barnes PJ: Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B₄. *Chest* 2003;123:1240–1247.
- 25 Milne ENC, Pistolesi M: Detecting and quantifying chronic bronchitis and emphysema; in Milne ENC, Pistolesi M (eds): *Reading the Chest Radiograph. A Physiologic Approach*. St Louis, Mosby, 1993, pp 267–310.
- 26 Miniati M, Filippi E, Falaschi F, Carrozzi L, Milne EN, Sostman HD, Pistolesi M: Radiologic evaluation of emphysema in patients with chronic obstructive pulmonary disease. Chest radiography versus high-resolution computed tomography. *Am J Respir Crit Care Med* 1995;151:1359–1367.
- 27 Sitkauskienė B, Sakalauskas R, Malakauskas K, Lotvall J: Reversibility to a β_2 -agonist in COPD: relationship to atopy and neutrophil activation. *Respir Med* 2003;97:591–598.
- 28 Kostikas K, Gaga M, Papatheodorou G, Karamanis T, Orphanidou D, Loukides S: Leukotriene B₄ in exhaled breath condensate and sputum supernatant in patients with COPD and asthma. *Chest* 2005;127:1553–1559.
- 29 Calverley PM, Burge PS, Spencer S, Anderson JA, Jones PW: Bronchodilator reversibility testing in chronic obstructive pulmonary disease. *Thorax* 2003;58:659–664.
- 30 Fabbri L, Pauwels RA, Hurd SS: Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD Executive Summary updated 2003. *COPD* 2004;1:105–141.
- 31 Confalonieri M, Mainardi E, Della Porta R, Bernorio S, Gandola L, Beghè B, Spanevello A: Inhaled corticosteroids reduce neutrophilic bronchial inflammation in patients with chronic obstructive pulmonary disease. *Thorax* 1998;53:583–585.
- 32 Yildiz F, Kaur AC, Ilgazli A, Celikoglu M, Kacar Ozkara S, Paksoy N, Ozkarakas O: Inhaled corticosteroids may reduce neutrophilic inflammation in patients with stable chronic obstructive pulmonary disease. *Respiration* 2000;67:71–76.
- 33 Keatings VM, Jatakanon A, Worsdell YM, Barnes PJ: Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* 1997;155:542–548.
- 34 Liu J, Sandrini A, Thurston MC, Yates DH, Thomas PS: Nitric oxide and exhaled breath nitrite/nitrates in chronic obstructive pulmonary disease patients. *Respiration* 2007;74:617–623.
- 35 Snoeck-Stroband JB, Lapperre TS, Gosman MM, Boezen HM, Timens W, ten Hacken NH, Sont JK, Sterk PJ, Hiemstra PS, Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease (GLUCOLD) Study Group: Chronic bronchitis sub-phenotype within COPD: inflammation in sputum and biopsies. *Eur Respir J* 2008;31:70–77.