

Are sputum eosinophil cationic protein and eosinophils differently associated with clinical and functional findings of asthma?

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Summary

Background Sputum eosinophil counts and eosinophil cationic protein (ECP) levels are usually increased in asthmatic patients. The correlation between sputum eosinophils or ECP and clinical findings of asthma has been previously investigated but many of these studies have been performed on small samples of asthmatic patients, considering only few clinical indices and often including patients on oral or inhaled corticosteroids, which might be confounding when interpreting the relationship between disease activity and airway inflammation.

Objective To assess whether sputum eosinophils and ECP were differently related to functional and clinical parameters of asthma in a large number of steroid-naïve asthmatic patients, taking into account several potential determinants of activity and chronicity of asthma.

Methods One hundred and twenty-nine patients with mild–moderate asthma were studied. Sputum was induced by hypertonic saline inhalation and processed using the whole sample method.

Results Sputum eosinophils and ECP significantly correlated with each other ($r = 0.41$, $P < 0.001$). When patients were grouped on the basis of high/low sputum eosinophils and high/low sputum ECP levels, significant differences were observed among groups, with patients with high sputum eosinophils and ECP showing the greatest asthma severity. In the overall sample, disease duration inversely correlated with sputum eosinophils, whereas FEV₁ and peak expiratory flow (PEF) inversely correlated with sputum ECP. Rescue β_2 -agonist use and total symptom score positively correlated with both eosinophil counts and sputum ECP. Stepwise regression analysis showed that symptom score and disease duration accounted for 17.6% of sputum eosinophil variance, whereas symptom score and FEV₁ accounted for 14.7% of sputum ECP variance.

Conclusions and Clinical Relevance Both sputum eosinophils and ECP are weakly related to clinical markers of asthma severity. However, ECP was more closely related to lung function parameters than eosinophil counts.

Keywords asthma, eosinophilic cationic protein, eosinophils, induced sputum

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Introduction

Asthma is a chronic inflammatory airway disease where eosinophils are potent effector cells [1]. Inflammatory cascade in asthma includes the recruitment and activation of eosinophils, which represent a marker of the inflammatory process activity [2]. These cells release inflammatory mediators such as eosinophil cationic protein (ECP), upon activation by several environmental

stimuli and intercellular adhesive interactions [3, 4]. ECP mediates several pathologic effects on bronchial epithelial cells, mucosal glands and airway smooth muscle [5].

Eosinophil cationic protein has been measured in blood, bronchoalveolar lavage and induced sputum, to evaluate its role as an additional marker of airway inflammation in asthma. Although serum ECP can be measured in the peripheral blood of asthmatic subjects

and shows some relationship with asthma severity and activity [6, 7], monitoring serum ECP offered no substantial advantage in the management of asthma when compared with blood eosinophils [8].

Several studies have shown that ECP can be found in induced sputum at different concentrations, according to the severity of the disease [9, 10]. In general, data on its relationship with sputum eosinophil levels are inconsistent, probably as a result of different degrees of cell activation [11–14]. The correlation between sputum eosinophils or ECP and clinical and functional findings has shown conflicting results, with some studies showing substantial equivalence between these two sputum markers [12], others reporting better correlation of ECP to pulmonary function and/or bronchial hyperresponsiveness [9, 11]. However, many of these studies, performed on small samples of asthmatic patients, considered only few clinical indices and often included patients on oral or inhaled corticosteroids, which might be confounding when interpreting the relationship between disease activity and airway inflammation. Indeed, corticosteroids have been reported to affect sputum ECP levels [14], thus making most correlations no longer significant [15]. Furthermore, the relationship between inflammatory markers and clinical or functional findings may be different according to whether indicators of disease 'activity' (current symptoms, exacerbation rate, rescue medication use) or 'chronicity' (disease duration, pulmonary function level) are considered [16].

We therefore studied a large number of corticosteroid-naïve, mild/moderate asthmatic patients to assess whether sputum eosinophils and ECP are differently related to functional and clinical indices of the disease, thus providing further clues in asthma management.

Materials and methods

Patients

We performed a retrospective analysis of the data obtained in 186 steroid-naïve patients with mild/moderate asthma recruited consecutively from the asthma clinic of the Cardiothoracic and Vascular Department, when they performed a global assessment of asthma for the first examination. The diagnosis of asthma was made according to internationally accepted criteria [17], in the presence of typical symptoms and risk factors, reversible airway obstruction and/or non-specific bronchial hyperresponsiveness to methacholine.

All patients were examined in a stable period of the disease, and patients with exacerbation of asthma in the last 4 weeks were excluded from analysis. None of the patients had received anti-inflammatory treatment in the last 3 months, and at the time of the study, all

patients had active symptoms and used bronchodilators as rescue medication. As part of our clinical routine, all patients performed (1) spirometry; (2) peak expiratory flow (PEF) monitoring and symptom score recording on a diary card for 2 weeks; (3) sputum induction with hypertonic saline (HS).

As additional measurements, all subjects underwent skin prick tests with 11 common airborne allergens (house dust mites, pollens, moulds, animal danders). Atopy was defined as at least one positive response (weal mean diameter: ≥ 3 mm, after subtracting negative control) to skin prick tests. Severity assessment was performed according to GINA recommendations, taking into account the frequency of day- and night-time symptoms, rescue medication use, PEF variability, limitation in daily life and FEV₁, % of predicted value [17].

The study has been performed according to the Declaration of Helsinki and notified to the Local Ethic Committee. Informed written consent was given by all patients as regard the use of personal data.

Peak expiratory flow, symptom evaluation, rescue β_2 -agonist use. Symptoms (wheezing, chest tightness, shortness of breath, cough) were rated on a scale from 0 (none) to 4 (never slept because of asthma) for night-time symptoms, and 0 (none) to 5 (could not attend to common daily activities because of asthma) for daytime symptoms, and added up to give the average daily symptom score [18]. Mean Morning PEF was expressed as percentage of predicted value. Rescue β_2 -agonist use was evaluated by recording the number of times the patients needed to inhale one or more puffs of short-acting β_2 -agonists. All patients recorded daily symptom score, use of β_2 -agonists as required and morning PEF on a diary card over a screening period of 2 weeks.

Methacholine challenge test. Methacholine (Sigma; St. Louis, MO, USA) was delivered by a jet nebulizer (DeVilbiss 646; DeVilbiss Healthcare; Somerset, PA, USA) using the procedure described elsewhere [19]. The cumulative dose of methacholine producing a 20% fall in FEV₁ (PD₂₀FEV₁) was computed; a value < 1000 μ g of methacholine was considered as positive for bronchial hyperresponsiveness (BHR).

Sputum induction and processing. After measurement of baseline FEV₁ and pre-treatment with 200 μ g inhaled salbutamol, hypertonic saline solution (NaCl 4.5% w/v) was delivered by means of an ultrasonic nebulizer (DeVilbiss Ultraneb 2000; DeVilbiss Healthcare, Somerset, PA, USA) with 2.8 mL/min output, as previously reported [20]. Sputum samples were processed using the whole sample method, as previously described [21]. The supernatants were stored at -80°C

for ECP measurement. At least 300 non-squamous cells were counted on cytopsin slides, and differential leucocyte counts were then expressed as percentage of total inflammatory cells, excluding squamous cells; samples with cell viability < 50% and cytopsin slides with an amount of squamous cells such that 300 inflammatory cells could not be counted were considered inadequate and therefore discarded. Because excessive sample dilution by salivary contamination, as expressed by squamous cell percentage, would affect inflammatory cell absolute counts and ECP measurements [22, 23]; according to a previous experience [24], we discarded sputum samples with $\geq 60\%$ squamous cells counted on cytopsin slides.

Eosinophil cationic protein levels in sputum supernatant were measured by means of UniCAP fluoroenzyme immunoassay (Pharmacia Upjohn AB, Uppsala, Sweden), as previously reported [20]. In our laboratory, the ECP intra-assay coefficient of variation is 4.5% and the inter-assay coefficient of variation is 8.2%; the detection limit of the ECP assay is 0.5 $\mu\text{g/L}$.

The upper limit of normal range for sputum eosinophils and ECP was set at 2% and 75 $\mu\text{g/L}$, respectively, derived from a group of 28 normal subjects enrolled in our laboratory. These values are very similar to those reported in other studies performed in normal subjects [9, 25] and were chosen as cut-off values.

Data processing and statistical analysis

Data normally or non-normally distributed were expressed as mean and standard deviation (SD), or median and range, respectively. Data with log-normal distribution were log-transformed; $\text{PD}_{20}\text{FEV}_1$ methacholine was expressed as a geometric mean (95% CIs). Data with normal distribution were analysed by one-way ANOVA with Student–Newman–Keuls multiple comparison test, while Kruskal–Wallis one-way analysis of variance followed by the *post hoc* Dunn's test was used for non-normally distributed data. Contingency table analysis using chi-squared tests was performed to compare categorical data. Single regression analysis was performed using Pearson's single correlation coefficient. Multiple regression analysis with stepwise selection of independent variables was carried out by relating sputum eosinophils or ECP (dependent variables) to clinical and functional indices, using different regression models. Sputum eosinophils, neutrophils and ECP, as well as $\text{PD}_{20}\text{FEV}_1$ methacholine, were log-transformed for single and stepwise regression analysis. Significance was accepted at the 95% level. Statistical data analyses were performed with statistical package (GraphPad Prism for Mac, version 5; GraphPad Software Inc., San Diego, CA, USA).

Results

A total of 27 of 186 selected patients did not collect an adequate sputum sample; furthermore, 30 of 159 patients showed a sputum sample with squamous cell percentages > 60%. All these 57 patients were not considered for the analysis. The characteristics of subjects analysed ($n = 129$) are reported in Table 1 and were not different from those of the subjects not included in the study (Table S1).

Mean FEV_1 was normal, although below 80% in 17 subjects (13.2%). One hundred and eleven of 129 patients underwent bronchial challenge test with methacholine, with 106 showing a positive response, as expressed by $\text{PD}_{20}\text{FEV}_1$ lower than 1000 μg ; however, the five patients with negative methacholine test were defined as asthmatics according to typical symptoms and reversible airway obstruction. Median daily symptom score and use of rescue medication were 1.1 (maximum value: 4.6) and 0.6 (maximum value: 5.2), respectively.

Mean cell viability of sputum samples was $73.9 \pm 10.6\%$ and mean percentage of squamous cells was $31.4 \pm 10.2\%$. Sputum eosinophils were > 2% in 65.1% of patients, whereas sputum ECP levels were > 75 $\mu\text{g/L}$ in 55.8% of patients.

Sputum eosinophils and ECP concentrations were significantly correlated with each other, both when eosinophils were expressed as percent of total inflammatory cells ($r = 0.41$, $P < 0.001$) and when expressed as absolute count/mL ($r = 0.46$, $P < 0.001$). There was, however, some data scattering, thus showing that the concordance between the two measurements was weak (Fig. 1).

The patients were then divided into 4 groups on the basis of sputum eosinophil and ECP values (i.e. higher

Table 1. Characteristics of asthmatic subjects

Sex, M/F	47/82
Age, years	37.8 ± 15
Smoking history, yes/no	23/106
Atopy, yes/no	87/42
Disease duration, years	8 (0.2–50)
Severity, mild/moderate	35/94
Daily β_2 -agonist, puffs/day	0.6 (0–5.2)
Daily symptom score	1.1 (0–4.6)
FEV_1 , % pred	89.9 ± 15.2
Mean PEF, % pred	77.0 ± 18.7
$\text{PD}_{20}\text{FEV}_1$, μg	123 (5–1294)

Age, FEV_1 and PEF are expressed as mean $\pm$ SD; disease duration, daily β_2 -agonist, daily symptom score are expressed as median (range); $\text{PD}_{20}\text{FEV}_1$ as Geometric mean.

FEV_1 , forced expiratory volume in 1 s; PEF, peak expiratory flow; $\text{PD}_{20}\text{FEV}_1$, provocative dose of methacholine causing a 20% fall in FEV_1 .

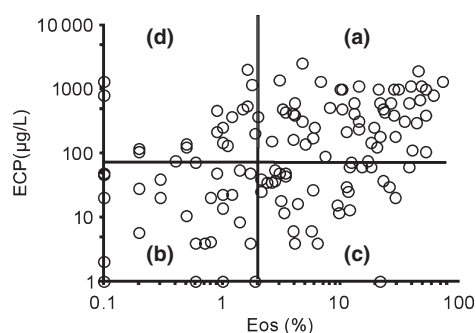


Fig. 1. Eosinophil percentages and ECP concentrations (in log scale) in sputum of steroid-naïve asthmatic patients. Horizontal and vertical lines show the cut-off values (normal upper limits) for ECP (75 µg/L) and eosinophils (2%), respectively. a: patients with eosinophils > 2% and ECP > 75 µg/L; b: patients with eosinophils ≤ 2% and ECP ≤ 75 µg/L; c: patients with eosinophils > 2% and ECP ≤ 75 µg/L; d: patients with eosinophils ≤ 2% and ECP > 75 µg/L. ECP, eosinophilic cationic protein.

or lower than the cut-off value): (a) 53 subjects with eosinophils > 2% and ECP > 75 µg/L; (b) 26 subjects with eosinophils ≤ 2% and ECP ≤ 75 µg/L; (c) 31 subjects with eosinophils > 2% and ECP ≤ 75 µg/L; (d) 19 subjects with eosinophils ≤ 2% and ECP > 75 µg/L. Functional and sputum indices measured in each group are shown in Table 2. In patients with high levels of both sputum eosinophils and ECP (Group a), the level of asthma severity, as expressed by lower FEV₁ values and higher symptom score and rescue medication use, was greater than both in patients with normal levels of both sputum eosinophils and ECP (Group b) and in patients with high sputum eosinophils but normal

sputum ECP (Group c). Furthermore, asthma severity in patients with high sputum eosinophils but normal sputum ECP values (Group c) was no different from that observed in patients with both sputum eosinophils and ECP levels within normal range (Group b). Finally, asthma severity in patients with high sputum ECP levels but normal sputum eosinophils (Group d) seemed to be intermediate between patients of Group a and those of Group b. Interestingly, Group d had the longest duration of disease among the four groups and showed FEV₁ and PEF similar to those of Group a.

All patients with high sputum ECP levels (Groups a + d) had longer duration of disease than patients with low ECP (Groups b + c), 10 (0.2–46) vs. 3 (0.2–50) years, $P < 0.05$. There were no differences in sex, age, smoking habits, atopy, bronchial hyperresponsiveness, sputum volume, cell viability (mean value in each Group: ≥ 71%), and absolute total inflammatory and squamous cell counts among the four groups (data not shown).

When all patients were considered, disease duration was inversely correlated with eosinophils, both when expressed as percentage and as absolute count/mL (Table 3). FEV₁% and PEF% were inversely correlated with sputum ECP but not with sputum eosinophil counts. Rescue β₂-agonist and total symptom score were positively correlated with both eosinophil counts and sputum ECP. There was no correlation between sputum markers and bronchial hyperresponsiveness. Furthermore, no significant relationship was found between absolute eosinophil counts or ECP levels and squamous cells (data not shown).

Table 2. Sputum inflammatory markers and clinical/functional findings measured in the four groups of patients divided according to EOS and ECP cut-off values

Characteristics of subjects	High sputum EOS and ECP (n = 53) (a)	Low sputum EOS and ECP (n = 26) (b)	High sputum EOS and low ECP (n = 31) (c)	Low sputum EOS and high ECP (n = 19) (d)								
					a vs. b	a vs. c	a vs. d	b vs. c	b vs. d	c vs. d		
Sex, M/F	25/28	9/18	7/24	7/12								
Age, years	36 ± 13.9	35.7 ± 13	37.6 ± 16.4	45.4 ± 16.7								
Atopy, yes/no	35/18	20/6	19/12	13/6								
Smoke, yes/no	9/44	5/21	6/25	5/14								
Disease duration, years	9 (0.2–40)	3.5 (1–50)	3 (0.2–40)	15 (1–46)								
Severity, mild/mod	4/49	14/12	11/20	6/13	‡	†	†					
FEV ₁ , % pred	84.7 ± 16.3	96.3 ± 13	93.7 ± 12	89.8 ± 15.3	†	†				*		
PEF, % pred	75.4 ± 16.9	85.2 ± 22	81.1 ± 15.8	71.9 ± 19	*							
Daily β ₂ -agonist	1 (0–4.6)	0.36 (0–1.9)	0.2 (0–1.6)	0.46 (0–5.2)	†	†						
Daily symptom score	1.6 (0–4.6)	0.9 (0–2.6)	1 (0–2.9)	1.4 (0–4)	†	*						
PD ₂₀ FEV ₁ , µg [§]	118 (5–1294)	166 (10–1199)	89.9 (10–1079)	146 (25–980)								

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

[§]Geometric mean.

Data are presented as No., median (range) or mean ± SD.

ECP, eosinophil cationic protein; EOS, eosinophils; FEV₁, forced expiratory volume in 1 s; mild/mod, mild/moderate; PEF, peak expiratory flow; PD₂₀FEV₁, provocative dose of methacholine causing a 20% fall in FEV₁.

Table 3. Correlations between sputum eosinophils or ECP and clinical or lung function parameters

Clinical and lung function parameters	Eosinophils (%)	Eosinophils (cells/mL)	ECP ($\mu\text{g/L}$)
Disease duration, years	$r = -0.22$; $P < 0.05$	$r = -0.21$; $P < 0.05$	ns
FEV ₁ , % pred	ns	ns	$r = -0.23$; $P < 0.01$
PEF, % pred	ns	ns	$r = -0.17$; $P < 0.05$
Daily β_2 -agonist	$r = 0.27$; $P < 0.01$	$r = 0.23$; $P < 0.05$	$r = 0.33$; $P < 0.001$
Daily symptom score	$r = 0.33$; $P < 0.001$	$r = 0.30$; $P < 0.001$	$r = 0.35$; $P < 0.001$
PD ₂₀ FEV ₁ , μg	ns	ns	ns

FEV₁, forced expiratory volume in 1 s; ns, nonsignificant; PEF, peak expiratory flow; PD₂₀FEV₁, provocative dose of methacholine causing a 20% fall in FEV₁.

Absolute counts of sputum neutrophils correlated positively with ECP ($r = 0.19$, $P < 0.05$) and with duration of asthma ($r = 0.18$, $P < 0.05$).

Because several clinical and functional variables were found to be associated with sputum eosinophils and/or ECP, multiple stepwise regression analysis was performed. The regression model included all the independent variables showing a significant correlation with sputum eosinophils or ECP in the single regression analysis, (disease duration, daily symptoms, FEV₁), excluding independent variables related to each other (in particular daily β_2 -agonist use, related to daily symptoms, and PEF, related to FEV₁) (Table 4).

Stepwise regression analysis showed that daily symptoms and disease duration predicted a proportion of the variance of sputum eosinophils ($R^2 = 17.6\%$, $P < 0.0001$) (Table 4), with daily symptom score giving the greatest contribution ($R^2 = 9.6\%$, $P < 0.001$). The

Table 4. Stepwise regression analysis of the relationship between sputum eosinophil percentages or ECP levels and clinical/functional indices. The independent variables are listed according to the order of inclusion in the regression models

Included variables in regression model	Global variance: adjusted R^2	Partial regression coefficient β (SE)	P -value
Eosinophils	0.176		< 0.0001
Daily symptom score		0.34 (0.059)	< 0.001
Disease duration, years		-0.29 (0.005)	< 0.01
ECP	0.147		< 0.0001
Daily symptom score		0.30 (0.072)	< 0.001
FEV ₁ , % pred		-0.19 (0.005)	< 0.05

ECP, eosinophilic cationic protein; FEV₁, forced expiratory volume in 1 s.

same results were obtained with eosinophils expressed as absolute counts (data not shown).

The results of stepwise regression for ECP showed that both daily symptoms and FEV₁% entered the model, representing significant independent determinants for ECP variance ($R^2 = 14.7\%$, $P < 0.0001$), although daily symptoms provided the greatest contribution ($R^2 = 11\%$, $P < 0.001$).

In both regression models, the inclusion of the variables related to each other, in particular daily β_2 -agonist use, related to daily symptoms, and PEF, related to FEV₁, gave similar results to those reported above (EOS: $R^2 = 17.5\%$, $P < 0.0001$; ECP: $R^2 = 14.7\%$, $P < 0.0001$); furthermore, by replacing the PEF and daily β_2 -agonist use with PD₂₀FEV₁ methacholine, which did not correlate with sputum markers in the single regression analysis but might potentially influence the regression, we obtained minor changes in the stepwise regression parameters, more evident in the ECP model (EOS: $R^2 = 16.7$, $P < 0.001$; ECP: $R^2 = 13\%$, $P < 0.001$).

Discussion

The aim of the present study, performed on a large number of corticosteroid-naïve asthmatic patients, was to evaluate whether sputum eosinophil counts and ECP concentrations are differently related to clinical and functional indices. We found that sputum eosinophil counts and ECP concentrations were both related to indices of asthma 'activity', such as current symptoms and rescue medication use, but differently related to indices of asthma 'chronicity': sputum eosinophil counts inversely correlated with the duration of the disease, whereas sputum ECP concentrations inversely correlated with pulmonary function, expressed as FEV₁ and PEF. This observation may suggest that these two markers of eosinophilic inflammation, although concordant in their relationship with current asthma symptoms (markers of 'activity'), may provide different clues about the chronic status of the disease, in terms of both disease duration and pulmonary function impairment.

In our study, the concordance between sputum eosinophil counts and ECP concentrations was mild. This fact may be expected, considering that ECP release from eosinophils depends on their activation status: few eosinophils, when highly activated by specific and/or increased degranulating agents, may release large amounts of ECP, whereas large numbers of eosinophils may be associated with low levels of ECP if not activated. As a matter of fact, it has been reported that patients with noneosinophilic airway disease, such as COPD or non-eosinophilic severe asthma, may have high sputum ECP levels; on the other hand, classic eosinophilic asthma may be associated with normal sputum ECP levels [9, 13]. All these observations

suggest that not only the number of eosinophils (as demonstrated by some interventional studies) [26] but also their activation degree (ECP levels) should be considered in the evaluation of asthma severity or activity. Therefore, both sputum eosinophils and ECP might play different roles as asthma inflammatory markers.

Eosinophil counts and ECP levels did not correlate with methacholine PD₂₀FEV₁ in our patients. Several discordant results have been reported about the association of eosinophilic markers to each other and with clinical and functional asthma indices. In a study, sputum ECP, but not serum ECP or sputum eosinophils, inversely correlated with FEV₁, but no correlation was found between the degree of sputum eosinophilia and sputum ECP levels [11]. One study showed that both sputum eosinophils and ECP correlate with airway hyperresponsiveness in asthmatic patients [27], whereas Gutierrez *et al.* [28] reported that only sputum eosinophil counts were inversely correlated with PC₂₀-methacholine in mild asthmatic patients. In a study comparing the relationship of blood and sputum eosinophils and ECP with clinical and functional indices of asthma severity, only sputum eosinophils and ECP were found to correlate with symptom score, spirometry and airway hyperresponsiveness, with stronger correlations for eosinophils than for ECP [12]; furthermore, although sputum ECP was reported to be a more sensitive marker of airway inflammation and asthma severity than serum ECP and blood eosinophils, the authors, however, concluded that sputum ECP adds no clinically important information to the results obtained with sputum eosinophils. On the other hand, some authors reported data suggesting that ECP may correlate better than eosinophils with pulmonary function and/or bronchial hyperresponsiveness, at least in subgroups of asthmatic patients [9], whereas others reported that both sputum eosinophils and ECP correlated with parameters of airway obstruction [12, 28, 29]. The discrepancy among these results might be explained by differences in the number of patients studied, in the levels of asthma severity, in the effects of inhaled corticosteroid treatment.

When our asthmatic patients were classified according to the levels of sputum eosinophils and ECP, the group with both high eosinophils and ECP had the highest daily symptom score and β_2 -agonist use, thus showing that both eosinophils and ECP were good markers of the current level of asthma severity. Conversely, we found low FEV₁ and PEF values only in the groups of patients with high ECP, suggesting that ECP may be more closely related to parameters of airflow obstruction than eosinophil counts. This concept was strengthened by the evidence of an inverse relationship between ECP, but not eosinophils, and FEV₁ or PEF in the entire sample of patients. Moreover, the stepwise

regression analysis confirmed that sputum eosinophil counts and sputum ECP concentrations may have different determinants. Our finding is in agreement with the results of previous studies showing that sputum ECP, but not sputum eosinophils, related to FEV₁ [9, 11].

According to these results, we might speculate that the symptomatic phases of the disease ('active phases') are associated with the recruitment of activated eosinophils (high sputum eosinophil counts and ECP levels) in the airways, whereas with the progression of the disease (long asthma duration and remodelling development), the number of eosinophils may decrease while still remaining strongly activated, thus explaining high ECP levels. In our study, changes in the pattern of airway inflammation with the increase in duration of disease were confirmed by the direct relationship between sputum neutrophil counts and asthma duration, as already reported [28], and between sputum neutrophil counts and ECP concentrations.

The different relationship of sputum eosinophils and ECP with clinical and functional findings may be interesting from a pathophysiologic point of view. However, considering the weakness of all correlations, and particularly the low variance associated with clinical and functional findings, the use of sputum eosinophils and ECP as predictors of clinical and functional findings is not advisable in routine asthma management.

Our study has some advantages over others previously published. First, the number of patients we studied is higher than that reported in previous studies [9, 10, 12]. Second, unlike in other studies, all our patients were corticosteroid-naïve to rule out the hypothesis that discrepancies between eosinophils and ECP might be due to their different sensitivity to corticosteroids. Actually, eosinophils are very sensitive to the effects of corticosteroids, and sputum eosinophilia is a good predictor of response to ICS; on the other hand, ECP seems more resistant to the effect of inhaled corticosteroids, as reported in studies on patients with asthma of different severity [30].

The present study was performed using the whole sample method for analysis of induced sputum, and it confirms and extends previous results obtained with the method of selected plugs [9, 10, 12]. It is important to point out that we discarded all samples with high percentages of squamous cells, which are considered as a marker of saliva contamination that may dilute sputum, thus making the measurement of soluble markers less reliable. Simpson *et al.* [23] reported that absolute cell counts and ECP levels, but not differential cell counts, significantly decreased when saliva added to aliquots of dispersed sputum reached a concentration of 70%.

In conclusion, our results demonstrate that sputum eosinophils and ECP are partly correlated with each

other, but differ in their relationship with clinical and functional findings of asthma. Sputum eosinophils seem to be related more to the active inflammatory process that may fade with time, whereas sputum ECP seems to be related more to the reduced lung function.

Conflict of interests

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of 57 asthmatic subjects not included in the study.

Table S1. Characteristics of 57 asthmatic subjects not included in the study

Sex, M/F	19/38
Age, yrs	39 ± 15,6
Smoking history, y/n	10/47
Atopy, y/n	35/22
Disease duration, yrs	6 (0.5-45)
Severity, mild/moderate	13/44
Daily β_2 -agonist, puffs/day	0.58 (0-3.5)
Daily symptom score	1.1 (0-4)
FEV ₁ , % pred	92 ± 13.8
Mean PEF, % pred	74.7 ± 16.4
PD ₂₀ FEV ₁ , µg	114 (10-1217)

Age, FEV₁ and PEF are expressed as mean±SD; Disease duration, Daily β_2 -agonist, Daily symptom score are expressed as median (range); PD₂₀FEV₁ as Geometric mean.

FEV₁: forced expiratory volume in 1 s; PEF: peak expiratory flow; PD₂₀FEV₁, provocative dose of methacholine causing a 20% fall in FEV₁.