Inflammation in chronic obstructive pulmonary disease: its assessment and the effects of corticosteroids
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Chapter 5

Inflammatory parameters and imminent exacerbations in COPD patients

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**Abstract**

During a two-months corticosteroid-free run-in period prior to a long-term intervention study, 8 of 29 patients with stable COPD experienced an exacerbation within 1 to 4 weeks after enrolment. We analysed retrospectively whether clinical and inflammatory parameters in blood and induced sputum at enrolment were associated with the imminent exacerbation.

At enrolment, cell differentials were obtained in induced sputum and blood, and markers of neutrophilic (myeloperoxidase: MPO; interleukin-8: IL-8) and eosinophilic (eosinophil cationic protein: ECP) inflammation and of airway permeability (quotient of \(\alpha\)-2-macroglobulin levels in sputum and serum: Q-A2M) were assessed. The patients had a mean age of 64 y, a mean FEV\(_1\) of 1.86 L (equivalent with 63 % of predicted), and showed a mean \(\beta_2\)-agonist reversibility of 4.4 % predicted.

GOLD stage 2 or 3 was a risk factor as 8 of 20 patients (40%) had an early exacerbation compared to none of 9 with stage 1 (p=0.01). Median blood eosinophil count was significantly higher in those with an exacerbation (p=0.03); a value above the median value for the entire study group was associated with a 13-fold increased risk of experiencing an exacerbation (95% C.I. 1.3 - 128, p=0.01). The four-fold higher median sputum eosinophil count (67 versus 15 x 10\(^3\)/g) approached significance (p=0.08). No significant differences were found for other inflammatory sputum parameters between patient groups. However, in 3 patients with an early exacerbation Q-A2M values and sputum ECP and MPO levels were at least 10-fold higher than for the other patients with an early exacerbation. Patients with early exacerbations showed either extensive or moderate inflammation, suggestive of two subgroups.

In conclusion, we hypothesize that, in addition to disease severity, high blood eosinophil counts and potentially also high sputum eosinophil counts are risk factors for an imminent exacerbation in patients with COPD.
Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory airways disease characterized by progressive and irreversible airway obstruction. COPD affects about 10% of the world population and its incidence is increasing.\(^1\) There is considerable interest in exacerbations of COPD, which reduce the patient’s health status for weeks to months and consequently constitute a major portion of health care costs.\(^2,3\) Furthermore, both the incidence of severe exacerbations and of hospitalisations for an exacerbation are associated with future hospitalisations and to mortality.\(^4-6\) In general, patients with more severe disease, patients who previously experienced exacerbations and those who were previously hospitalised for an exacerbation are reported to most likely experience additional exacerbations.\(^4\) Long-term treatment with inhaled corticosteroids was found to significantly reduce exacerbation frequency in moderate to severe COPD, be it with only a modest effect on the progression of airway obstruction.\(^7-12\) The mechanisms underlying this effect of corticosteroids on exacerbation frequency is unknown, particularly as the hallmark of airway inflammation in COPD, neutrophilic inflammation, is considered less responsive to corticosteroid treatment.\(^13-15\) Corticosteroids are however known to inhibit eosinophilic inflammation, which may be relevant for COPD, as sputum eosinophilia during COPD exacerbations has been reported.\(^16,17\) In a longitudinal study, investigating the long-term effects of corticosteroids on parameters of airway inflammation in blood and induced sputum and effects on clinical indices and exacerbations,\(^18\) patients with stable COPD were enrolled in a 2-months run-in period without corticosteroid treatment. We observed a high incidence of exacerbations within one month after enrolment. To provide a clue as to the underlying pathophysiological mechanism leading to these exacerbations, we analysed retrospectively whether patient characteristics and/or inflammatory parameters as assessed at enrolment in induced sputum and in blood were associated with the occurrence of an early exacerbation.

Methods

Subjects

Patients had clinically stable, smoking-related COPD. In line with the GOLD criteria, candidate patients were aged 40-75 years at entry, had middle-age onset of symptoms, a cigarette consumption of at least 15 Pack-Years, a pre-bronchodilator FEV\(_1\) > 1.0 L and >30% of predicted and a post-bronchodilator FEV\(_1\)/VC ratio smaller than 0.70.\(^1,19\) Reversibility in FEV\(_1\) was below 11% of predicted.\(^19\) Excluded were patients with a history of asthma, a known allergy, those with a positive blood RAST test to four common allergens or a known \(\alpha\)-1-antitrypsin deficiency. For safety reasons, patients with unstable or serious concomitant diseases and (potential) pregnancy were excluded. Written
informed consent was obtained from all patients and the study was conducted according to the declaration of Helsinki and after approval by the local medical ethics committee.

Study design
Within one week after enrolment a medical examination, a sputum induction procedure and extensive pulmonary function tests were performed. Reversibility was assessed after inhaling 4 puffs of 0.25 mg of the $\beta_2$-agonist terbutaline via a spacer device. Dyspnea was assessed with the Borg dyspnea score, ranging from 0 to 10. Symptom severity and health status was assessed with the Clinical COPD Questionnaire (CCQ), ranging 0 to 6. The patients were withdrawn from the use of corticosteroids and were asked to return for a second visit after two months. They were supplied with written instructions on allowed (bronchodilators) and disallowed (corticosteroids) bronchopulmonary medication. An exacerbation of COPD was defined according to Burge et al., as an increase in symptoms, necessitating treatment with systemic corticosteroids, with or without antibiotic treatment.

Sputum induction and analysis of sputum and peripheral blood
After prior inhalation of the $\beta_2$-agonist, sputum was induced by nebulizing hypertonic saline, 3%, 4% and 5% each during 5 minutes. After careful mouth-rinsing and nose-blowing, sputum was collected and kept on ice, weighted and liquefied with dithiotreitol. Sputum cells were collected by centrifugation, counted and cytospins were prepared for differential counts in minimally 500 non-squamous cells. The liquid phase was aliquoted and stored at -80°C until further analysis. An EDTA blood sample was taken and serum was stored in aliquots at -80°C.

Soluble inflammatory markers were chosen to represent neutrophilic inflammation (myeloperoxidase (MPO) and interleukin (IL)-8), eosinophilic inflammation (eosinophil cationic protein (ECP)) and airway permeability (the ratio of $\alpha$-2-macroglobulin (A2M) levels in sputum and in serum and the same ratio for albumin (Alb): Q-A2M and Q-Alb respectively). A2M, ECP, IL-5, IL-8 and MPO were determined with ELISA. Alb was determined using an immunoturbidometric analysis. Cell counts from sputum samples with 80% or more squamous cells were excluded from the statistical analyses.

Statistical analysis
Continuous variables were compared using a t-test or Mann-Whitney U test as appropriate, categorical variables using the Fisher’s exact test. Due to non-normal distribution of sputum data, which remained after logarithmic transformation, all inflammation data were compared between groups using non-parametric tests. Positive sputum eosinophilia was defined as a sputum % eosinophil count of 3.0% or more. Odd Ratios were calculated and presented with 95% confidence interval (C.I.). SPSS-PC version 12 was used for the statistical analyses and two-sided p-values $\leq$ 0.05 were considered statistically significant.
Inflammatory parameters and imminent exacerbations

The sample size of the study population was determined for the parent study, the present analysis was performed post hoc.

Results

Exacerbations.

Twenty-nine clinically stable patients with mild to severe COPD (Table 1), 20 of whom used inhaled corticosteroids (ICS) in a dose ranging from 0.2 to 1.6 mg daily, were enrolled into the 2-months run-in period and refrained from corticosteroid treatment. Eight patients, 7 after withdrawal of their ICS and 1 without previous ICS-treatment, developed an exacerbation after 7 to 29 days (mean 20 days) of the 60 days run-in period.

Patient characteristics

The demographic and other characteristics at baseline for the 8 patients with an early exacerbation and the 21 patients without an exacerbation are shown in Table 1. Based on previous studies, it was to be expected that patients with more severe COPD according to the degree of airway obstruction had a higher risk of experiencing an early exacerbation. Differences in FEV$_1$ and FEV$_1$/VC between the two groups were indeed statistically significant ($p=0.04$ and $p=0.05$ respectively), whereas the difference in FEF$_50$ almost reached significance ($p=0.07$). None of the 9 patients with GOLD stage 1 (i.e. FEV$_1$ above 80% of predicted) had an exacerbation in contrast to 3 (27.3%) of the 11 patients with GOLD stage 2 and 5 (55.6%) of the 9 patients with GOLD stage 3 (Figure 1). As a consequence, patients with GOLD stage 2 or 3 had a significant higher chance of experiencing an exacerbation than those with stage 1 (OR 1.7, 95% C.I. 1.2 – 2.4, $p=0.026$). Other indices of disease severity like the Borg dyspnoea score and the health status, assessed with the CCQ showed only a trend towards more severe disease in those with an early exacerbation.

Figure 1. Proportion of patients with GOLD stage 1, 2 and 3 experiencing an exacerbation within 2 months after being enrolled in the study.
We also anticipated that patients who were taken off ICS would have a higher risk of developing an early exacerbation since the ICS treatment was to be stopped.\textsuperscript{29,30} There was a four-fold higher risk for an early exacerbation in patients using ICS until enrolment in the study, although this did not reach statistical significance (OR 4.3, 95\% C.I. 0.44 – 41.8, \(p=0.20\), Figure 2). For no other demographic or clinical parameter an association with an early exacerbation was found.

Inflammatory parameters

Inflammatory parameters determined in induced sputum and in peripheral blood are summarized in Table 2. In 5 of the 29 sputum samples (of four patients without and one subject with an exacerbation) the sputum squamous cell count was > 80\%, leading to exclusion of the sputum data from the statistical analyses.
### Table 2. Inflammatory parameters in sputum and peripheral blood from patients at the start of the observation period, and presented separately for those with and without an early exacerbation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N=8 with</th>
<th>N=21 without</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induced sputum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cell Count (10^6 /g)</td>
<td>1.63 (1.28-6.10)</td>
<td>2.0 (1.2-3.3)</td>
<td>0.92</td>
</tr>
<tr>
<td>Neutrophil count (10^6 /g)</td>
<td>1.3 (1.0-4.7)</td>
<td>1.4 (0.9-2.2)</td>
<td>0.73</td>
</tr>
<tr>
<td>Eosinophil count (10^3 /g)</td>
<td>67 (8-373)</td>
<td>15 (6-26)</td>
<td>0.08</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>80.8 (66.8-97.2)</td>
<td>76.0 (61.5-86.2)</td>
<td>0.41</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.6 (0.5-11.2)</td>
<td>0.6 (0.2-2.3)</td>
<td>0.21</td>
</tr>
<tr>
<td>MPO (μg/g)</td>
<td>9.1 (2.9-152.3)</td>
<td>16.4 (3.6-36.9)</td>
<td>0.70</td>
</tr>
<tr>
<td>IL-8 (ng/g)</td>
<td>3.9 (1.2-47.5)</td>
<td>5.0 (2.7-27.2)</td>
<td>0.92</td>
</tr>
<tr>
<td>ECP (μg/g)</td>
<td>0.39 (0.10-25.3)</td>
<td>0.33 (0.07-1.15)</td>
<td>0.44</td>
</tr>
<tr>
<td>Albumin (μg/g)</td>
<td>130 (15.8-873)</td>
<td>25 (6.5-131)</td>
<td>0.33</td>
</tr>
<tr>
<td>α-2-Macroglobulin (μg/g)</td>
<td>3.8 (0.8-35.4)</td>
<td>1.2 (0.8-2.8)</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Peripheral blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucocyte count (10^9 /L)</td>
<td>7.8 (6.1-9.4)</td>
<td>7.9 (6.5-8.5)</td>
<td>0.94</td>
</tr>
<tr>
<td>Eosinophil count (10^5/L)</td>
<td>219 (207-273)</td>
<td>130 (83-201)</td>
<td>0.029</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>56.9 (47.3-68.3)</td>
<td>60.9 (54.8-67.5)</td>
<td>0.38</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>3.4 (2.1-4.3)</td>
<td>2.0 (1.1-2.9)</td>
<td>0.045</td>
</tr>
<tr>
<td>Albumin (mg/ml)</td>
<td>43.1 (42.1-47.1)</td>
<td>43.8 (40.5-45.4)</td>
<td>0.63</td>
</tr>
<tr>
<td>α-2-Macroglobulin (mg/ml)</td>
<td>2.95 (2.23-4.30)</td>
<td>2.61 (2.37-3.39)</td>
<td>0.56</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>4.7 (&lt;1 – 5.9)</td>
<td>1.2 (&lt;1 – 6.6)</td>
<td>0.76</td>
</tr>
<tr>
<td>CRP (mg/ml)</td>
<td>3.5 (2.3 – 9.5)</td>
<td>4.0 (2.0 – 9.5)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Permeability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q-Albumin (x 1000)</td>
<td>3.1 (0.3-18.8)</td>
<td>0.57 (0.15-2.9)</td>
<td>0.29</td>
</tr>
<tr>
<td>Q- α-2-Macroglobulin (x 1000)</td>
<td>1.2 (0.3-11.1)</td>
<td>0.42 (0.33-1.20)</td>
<td>0.24</td>
</tr>
<tr>
<td>RCE</td>
<td>0.69 (0.33-1.46)</td>
<td>0.85 (0.23-2.35)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Data expressed as median (Inter Quartile Range); p-values from Mann-Whitney tests, bold printing p values <0.05; MPO: myeloperoxidase; ECP: eosinophil cationic protein; IL-8: interleukin-8; Q-values are the quotient of sputum / serum levels of albumin and alpha-2-macroglobulin; RCE: relative coefficient of excretion, the quotient of Q-alpha-2-macroglobulin / Q-Albumin.
Patients with an imminent exacerbation in comparison to those without an exacerbation showed higher median values for many parameters, especially eosinophilic markers, but not for neutrophilic markers. However, only the difference in sputum eosinophil count tended to statistical significance (p=0.08). Sputum eosinophil counts above 3.0 % tended to be associated with an early exacerbation, but this was not statistically significant (O.R. 5.6, 95% C.I. 0.7 – 46, p=0.12). In line with sputum eosinophilia, in these patients with an early exacerbation, there was a statistically significant 1.5-fold higher median absolute and relative eosinophil count in peripheral blood of the patients who experienced an early exacerbation (p=0.028 and p=0.047, respectively; Figure 3). A peripheral blood eosinophil count above the calculated median value of 160x10^6/L for the entire group of 29 patients was associated with an Odd Ratio of 13.0 to experience an early exacerbation (95% C.I. 1.3 – 128, p=0.01). It must be noted that peripheral blood eosinophil counts for all patients were within the normal range. There were no differences in the serum levels of IL-8 and C-Reactive Protein (CRP).

As IL-5 is considered a pro-eosinophilic factor, we also assessed plasma IL-5 levels but found no detectable levels of IL-5 in plasma in patients from either group.

It may be argued that the extent of inflammation rises just before an exacerbation becomes clinically manifest. However, within the group of eight patients with an early exacerbation there was no sign of a more extended inflammation in those with the shortest interval until the exacerbation.

The median values for cell counts and for most of the soluble parameters were similar in the group of patients who experienced an exacerbation, but the range was large. The latter was a result of a non-homogeneous and apparently dichotomous distribution of these parameters in this group (Figure 4). For three of the eight patients with an imminent exacerbation we found high levels of MPO and particularly of ECP, as well as increased values of Q-A2M. In contrast, the levels of these parameters in the other patients with an imminent exacerbation were in the lower range of the values observed for the patients without exacerbation.

**Figure 3.** Peripheral blood eosinophil count (10^6/L) for enrolled COPD patients. Open symbols represent patients who stayed exacerbation-free for 2 months, filled (grey and black) symbols represent subjects who experienced an exacerbation within one month, p=0.030. The grey-filled symbols represent patients with relatively high levels of inflammatory markers in sputum.
Inflammatory parameters and imminent exacerbations

Although patients were enrolled in the study during a clinically stable phase of their disease, 8 of 29 COPD patients experienced an exacerbation requiring medical intervention within four weeks of a corticosteroid-free two months’ follow-up period. This prompted us to retrospectively analyse demographic, clinical and inflammatory parameters at enrolment in relation to these early exacerbations. We observed significantly higher blood eosinophil counts for the group of patients with an imminent exacerbation and a trend towards higher sputum eosinophil counts. Interestingly, we found that levels of most inflammatory markers in induced sputum did not significantly relate to the occurrence of an early exacerbation.

In our study, patients with more severe disease as based on lung function criteria, i.e. GOLD stage 2 or 3, had a significant 1.7-fold higher chance of experiencing an early exacerbation than patients with milder disease. Also, patients who used ICS prior to the study had a (non-significant) 4-fold higher chance of experiencing an exacerbation as compared to those not using ICS. These findings are in line with other studies into COPD. Especially the re-analysis of the ISOLDE study indicated a lower FEV₁ and previous ICS use as risk factors for early exacerbations after ICS withdrawal, while additional studies pointed also to low FEV₁, higher reversibility and systemic levels of fibrinogen or serum Amyloid A being related with early exacerbations.

In the present study, patients with asthma were carefully excluded; only older subjects with a significant smoking history, an abnormal FEV₁/VC ratio, limited reversibility and absent allergy were included. Therefore, we consider the present findings not biased by

Figure 4. Concentration of MPO (μg/g), ECP (ng/g), IL-8 (ng/g) in induced sputum and the value of Q-A2M (x1000). Note the logarithmic scale. Open symbols represent patients who stayed exacerbation-free for 2 months, filled (grey and black) symbols represent subjects who experienced an exacerbation within one month. The grey-filled symbols represent the 3 patients with relative high levels of all inflammatory markers in sputum.

Discussion
underlying allergy or asthma and thus representative of COPD, despite the associations with for eosinophil counts.

The current results indicate that stable COPD patients prior to an exacerbation have relatively high blood eosinophil counts and maybe also increased sputum eosinophil counts. The mechanisms underlying these high eosinophil counts is unknown, but at least it does not relate to elevated (detectable) systemic IL-5 levels. Furthermore, there seems to be no strong relation between the high eosinophil counts and the extent of airway inflammation, particularly sputum ECP levels, indicating that the high eosinophil numbers are not related to airway inflammation per se. A possible explanation is that patients with intrinsically high circulating eosinophil numbers may develop a more profound eosinophilic airway inflammation upon an appropriate trigger for an exacerbation. This is in line with some earlier studies, where sputum eosinophilia was observed during COPD exacerbations, although this was not confirmed in a later study. It is tempting to speculate that, at least in some COPD patients, ICS treatment prevents escalation of eosinophilic airway inflammation and so may reduce exacerbation frequency. In the present study, no sputum was collected during the exacerbations and thus we can not clarify whether eosinophilic airway inflammation increased prior to the exacerbation. A recent study in which treatment was titrated directed at low sputum eosinophil counts resulted in a lower incidence of exacerbations than during regular treatment of COPD.

An earlier reported association between high sputum IL-8 levels and a higher frequency of COPD exacerbations, led us to hypothesize that airway inflammation in stable disease is more pronounced in patients experiencing an early exacerbation, who may also be the patients with more frequent exacerbations. Additionally, some studies reported a decrease in the neutrophilic inflammation markers MPO and IL-8 by corticosteroid treatment. In the present study median numbers of inflammatory cells and median levels of most soluble markers, including IL-8, were similar in the groups that did or did not exacerbate shortly hereafter. Solely the eosinophil numbers and parameters of airway permeability showed more extended inflammation in those with an imminent exacerbation. But due to the large intra-individual variation, the differences with the group that did not exacerbate did not reach statistical significance. For 3 out of these 8 patients, however, we showed markedly increased levels of MPO and ECP and values of Q-A2M. The levels for most of the inflammatory parameters measured in sputum from these 3 patients were very high compared to that for patients not having an early exacerbation. The remaining patients with an early exacerbation had levels of these parameters in the lower range of those found for patients without an early exacerbation. These findings are suggestive of two subpopulations of patients with an imminent exacerbation: one with high levels of inflammatory parameters and one with levels, similar to those for patients with COPD without an imminent exacerbation. Whether the cause of these exacerbations (only corticosteroid withdrawal or a microbial infection)
or whether the clinical course differed between these apparent subpopulations is unknown. Larger studies are needed to clarify this. Several studies investigated systemic inflammation and its relation to imminent COPD exacerbations and suggested that levels of fibrinogen, serum Amyloid A or CRP may be linked to upcoming exacerbation or recurrence of exacerbations. In the present study no such association was found for serum IL-8 and CRP.

Sputum was not frozen before liquification and aliquoting, and thus ECP and MPO in the soluble phase reflect activation of eosinophils and neutrophils, respectively, rather than the number of cells present. Inflammation leads to damage of the airway mucosa and consequently to an increased exudation of serum proteins such as albumin and A2M into the airway lumen. Due to its molecular size, leakage of A2M into the airway lumen is low in non-inflamed airways and increases during inflammation. An increased presence of A2M in sputum and a high quotient of the concentrations of A2M in sputum over serum indicate increased airway permeability. Thus, the 3 patients with an imminent exacerbation and with high levels of ECP and MPO and high values for Q-A2M, had a prominent inflammatory process in their airways, despite inhaled corticosteroid treatment, suggestive of more extensive local damage than in the other COPD patients with or without an imminent exacerbation. In fact, the other patients with an imminent exacerbation, including the only exacerbating patient without prior inhaled corticosteroid treatment had levels of ECP and MPO and values for A2M in the low range of those for patients with no early exacerbation, suggestive of a milder airway inflammation and limited local damage prior to the exacerbation.

IL-8 is a potent chemoattractant of neutrophils and under certain conditions also of eosinophils, and thus IL-8 can drive neutrophilic and eosinophilic inflammation. The apparent dichotomy between inflammatory parameters for the two subpopulations of patients with an imminent exacerbation is also seen for IL-8 levels in sputum, suggestive of a key role for IL-8 in driving inflammation for both subgroups. Levels of IL-8 and those for the other inflammatory markers were found in samples that were collected only at the time that the majority of the patients were still using ICS, albeit with unknown compliance and different doses. Therefore, we can not assess whether and, if so, in which patients there was an effective inhibition of IL-8 production by ICS which was found in other studies. It would be of interest to determine whether IL-8 levels increase upon withdrawal of ICS in the patients with an imminent exacerbation.

In conclusion, despite its small size, the present study indicates that relatively high eosinophil counts in blood and sputum in clinically stable patients with COPD are related to an enhanced risk of developing an exacerbation. The extent of airway inflammation in stable disease per se did not correlate with the occurrence of an early exacerbation, but the large differences in airway inflammation between individual patients with
an imminent exacerbation are suggestive of two subgroups of patients with an early exacerbation. These findings need to be addressed in a larger and prospective study with frequent sputum sampling, possibly hereby allowing the identification of patients at risk of developing an exacerbation.

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References


