3

TIMECOURSE OF INFLAMMATORY MARKERS AND AT EXACERBATION OF COPD

3.1 Introduction

COPD exacerbations are associated with increases in airway and systemic inflammation that are heterogeneous and probably related to the aetiology of the exacerbation. In Chapter 1 we reviewed the data available on the time course of inflammatory and infective changes at exacerbation of COPD available prior to the current study. We established the data was limited and included a very selected subgroup of COPD patients.

Exacerbations have a tendency to cluster in an individual as demonstrated by studies of patients hospitalised with severe COPD exacerbations (Roberts et al 2002, Price et al 2006). Such studies have shown that after the index exacerbation, patients are at increased risk of readmission and one study showed that 34% of patients were readmitted with a recurrent exacerbation in the 3 months following their discharge. Frequent readmissions with COPD exacerbations have been highlighted as an independent risk factor for increased mortality (Soler-Cataluna et al 2005). However, it was not known whether the recurrent exacerbations were associated with persistence of a heightened inflammatory status or were related to non-recovery of the index exacerbation.

3.2 Aim

Our aim was to conduct a prospective study in a well-characterized cohort of COPD patients and assess the time course of airway and systemic inflammation in the stable state, at exacerbation and during the recovery at day 7, 14 and 35. We wanted to assess the differences in the airway and systemic inflammatory markers between frequent and infrequent exacerbators, patients with recovered and non-recovered exacerbations and in patients with different severities of COPD and those with and without symptoms of a common cold at onset. We also set out to assess the relationship between recurrent exacerbations occurring within 50 days of the index exacerbation and the airway and systemic inflammation during the index episode.

We also assessed the differences in symptoms, airway and systemic inflammation in patients treated with and without oral steroids. Finally we assessed the factors related to hospital admissions in our group of COPD patients.

3.3 Methods

3.3.1 The London COPD Cohort

The London COPD Cohort is a prospective observational cohort of patients with COPD running since 1995. It was established to investigate the nature and mechanisms of COPD exacerbations. Patients are followed up with daily diary cards where they record any increase in their respiratory symptoms and their morning peak expiratory flow rate (PEFR). Patients also recorded the number of hours spent outdoors and any change to their COPD treatment. They are seen in the study clinic routinely every 3 months and whenever they experience an exacerbation. Patients have direct access to the study team via a dedicated phone line.

3.3.2 Protocol of the time course study

Patients are recruited in the stable state, when they are at least six weeks away from their last exacerbation. They are then followed prospectively with daily diary cards till they experienced an exacerbation.

At exacerbation patients are generally seen the same day or during the weekend within 48 hours of them contacting the study team. Their symptoms are reviewed and the exacerbation is confirmed by the study physician. Spirometry is recorded and samples of sputum (spontaneously produced or induced) and peripheral venous blood are taken, prior to initiation of treatment. No patients take oral steroids or antibiotics prior to their visit to the study clinic with an exacerbation.

Patients are treated with antibiotics and/or oral steroids according to the clinical severity of the episode. The treatment is left to the discretion of the attending physician and usually includes antibiotics and/or oral steroids as well as increased bronchodilators.

Patients are then asked to come back after 7, 14 and 35 days. At each of these subsequent visits, the symptoms are reviewed, spirometry is recorded and samples of blood and sputum obtained. Additional treatment is prescribed depending on the clinical evolution of the episode.

3.3.3 Choice of airway and systemic inflammatory markers

In this study we assessed airway inflammation by measuring the levels of IL-6 and IL-8 in the sputum. The systemic inflammation was assessed by measuring the levels IL-6 and CRP in the serum. IL-6 was chosen as it can be consistently and repeatedly measured in the sputum and in the blood. CRP was chosen as it can be easily measured at low cost in many health care settings. Sputum IL-8 was chosen as it is a marker of the neutrophilic inflammation observed in COPD and it can be measured with commercially available kits. IL-6 in the sputum and serum and IL-8 in the sputum were measured with an enzyme linked immunosorbent assay (ELISA). Serum CRP was measured in our hospital laboratory with a luminometric analyzer.

3.3.4 Patient recruitment and follow up

Patient recruitment

At the beginning of the time course study, 66 patients were recruited from the London COPD cohort. These patients had severe to very severe COPD. Patients with mild and moderate COPD were recruited from primary care and the Outpatient's department of the London Chest Hospital. Between April 2003 and September 2004 150 patients were recruited for this study. In total 80 time courses were sampled in 80 different patients between April 2003 and March 2005. 73 of those time courses have been analysed and will be reported in the rest of this text.

The inclusion criteria for the time course study were a history of at least ten pack year's current or past smoking history and a FEV_1/FVC ratio below 70. They also had to have less than 15% or 200mls reversibility to beta-2 agonists. Patients with a

history of asthma, significant bronchiectasis, carcinoma of the bronchus or other significant respiratory diseases were excluded, as well as those unable to complete daily diary cards.

At the recruitment visit which took place in the outpatient department of the London Chest Hospital, a detailed history was taken, the patient's medication was recorded and a clinical examination was performed including weight and height. Lung function was performed and reversibility to beta-2 agonists was assessed with Salbutamol 400 mcg administered via a volumatic spacer. Lung function measurement were taken at least 4 hours after the last dose of short acting beta-2 agonist, and 12hrs after their last dose of long acting beta-2 agonist. Partial pressures of arterial oxygen and carbon dioxide were measured on arterialised ear lobe blood gases (measured on a Model 278 Blood Gas Analyser, Ciba-Corning, Medfield, USA). The patients were also asked to complete the St Georges Respiratory Questionnaire and provide a sample of peripheral venous blood and spontaneously produced sputum. At the recruitment visit all patients had their full blood count, urea and electrolytes, liver function test, albumin, calcium levels and C-reactive protein measured in the hospital laboratory.

Patient follow-up

All patients were instructed on how to keep daily diary cards on which they recorded their morning post medication peak expiratory flow rate (PEFR) with a mini-Wright peak flow meter (Clement Clarke International Ltd, Harlow, UK) and any increase in their daily respiratory symptoms.

The respiratory symptoms were alphabetically coded, in the following way:

A = increased dyspnoea

B1 = increased sputum volume

B2 = increased sputum purulence

C = symptoms of a common cold

D = chest tightness or wheeze

E1 = sore throat

E2= increased cough

F = fever

An example of a diary card is shown in the **Appendix 1**.

Patients were routinely seen every 3 months in the research clinic, their diary cards were reviewed and spirometry was recorded.
They were asked to contact the study team via a dedicated phone line when they experienced any increase in their daily respiratory symptoms. They were generally seen within 48 hours of the onset of symptoms by a physician from the study team; their symptoms were reviewed and the exacerbation confirmed according to our symptomatic definition and patients were sampled before starting any treatment.

3.3.5 Definition of an exacerbation

The diagnosis of an exacerbation was based on symptomatic criteria previously validated by our group. Increased dyspnoea, increased sputum volume and increased sputum purulence were called major symptoms; nasal congestion/discharge, sore throat, increased chest tightness/wheeze or increased cough were termed minor symptoms. An exacerbation was defined as any two major symptoms or one major and one minor symptom, present for at least two consecutive days, the first of which was called the day of onset of the exacerbation. Symptoms recorded continuously in the five days before exacerbation onset were discounted when deciding whether an exacerbation had occurred or not.

3.3.6 Definition of exacerbation severity

Each symptom, major and minor, was binary coded as 1 (present/ increased over baseline) or 0 (absent/ not increased) and then summed. This symptom score was calculated daily throughout the exacerbation. The exacerbation severity was assessed by using the change at onset of exacerbation in the total symptom score and the percentage drop in FEV_1 compared to baseline.

3.3.7 Definition of exacerbation frequency

The number of exacerbations experienced by each patient between April 2002 and December 2004 was determined and the median exacerbation frequency was calculated for this study. Patients with an exacerbation frequency above the median for the study were called frequent exacerbators, and those with an exacerbation frequency below the median were called infrequent exacerbators.

3.3.8 Definition of recovery

The recovery time for symptoms was defined as the time from onset of the exacerbation to the day on which a three day moving average of the symptom score returned to baseline (defined as the median symptom score recorded on days 8 to 14 prior to the onset of the exacerbation). The PEFR recovery time was defined as the time from exacerbation onset to the day on which a three day moving average of PEFR returned to baseline (defined as the mean PEFR recorded on days 8 to 14 prior to the onset of the exacerbation).

3.3.9 Patient sampling

Samples were taken at baseline and at exacerbation, prior to treatment, when patients were not taking any antibiotics or oral steroids for at least 6 weeks. After the exacerbation visit they were reviewed after 7, 14 and 35 days during the recovery. Forty nine prospective baseline samples were collected a median (IQR) 160 days (12-578) before the exacerbation, 15 baseline samples were collected a median(IQR) 141 days (50-374) after the exacerbation when patients were clinically stable. Baseline samples were not available for nine patients.

Spirometry

All the lung function indices were measured with a rolling seal spirometer (SensorMedics Ltd, Yorba Linda, California, USA). All patients had baseline FEV_1, FVC and PEFR measured in the stable state, at least four hours after their morning

medication, which included beta-2 agonists. All the baseline lung function data was generally recorded between 9am and noon.

At exacerbation, patients FEV_1, FVC and PEFR was measured between 9am and noon, at least 20 minutes after their last dose of short acting beta-2 agonist.

Sputum

Sputum samples were collected spontaneously and if no spontaneous sputum was available patients were induced with hypertonic saline. Our group has previously demonstrated that the inflammatory markers are equivalent in spontaneous and induced sputum (Bhowmik et al 1998). The sputum induction was performed by nebulisation with 3% saline using a DeVilbiss UltraNeb 2000 ultrasonic nebuliser. Patients were pre-medicated with Salbutamol 200 mcg given via a volumatic spacer 10 minutes prior to nebulisation. Nebulisation was performed for 7 minutes at first, followed by oxygen saturation and spirometry measurements. If the patient had not produced an adequate sputum specimen and if the FEV_1 had not fallen by more than 20%, nebulisation was repeated for another 7 minutes. Patients were instructed to rinse their mouth with water and blow their noses before expectorating sputum into a sterile pot. Sputum was collected in the morning, kept at room temperature and was processed within 2 hours of collection.

Peripheral venous blood

All patients had 7 mls of peripheral venous blood collected via a Vacutainer system into a sterile serum sampling tube (BD Vacutainer ® spray coated silica tube) and 5mls of blood taken into a sterile Citrate tube (BD Vacutainer ® 3.2% buffered sodium citrate tube) . Blood samples were kept at room temperature and processed within 2 hours of collection to prevent degradation of any cytokines.

3.3.10 Samples processing

Sputum processing

The sputum was separated from contaminating saliva using a pair of disposable forceps and the weight of the sputum recorded. A selected portion of sputum was placed in a pre-weighted tube and the weight of the portion of sputum was recorded.

The sputum portion was mixed with 9 times it weight of phosphate buffered saline (PBS) and a few siliconised glass beads. The tube was agitated till the sputum was homogenised, then the mixture was filtered through a 50µm nylon gauze to remove thee mucus and debris. The supernatant was decanted and aliquots of 500 µl were stored at -80 ºC until analysis.

Blood processing

The whole blood was centrifuged at 2000 rpm for 10 min at 4 ºC to separate the serum. 500 µl aliquots of serum and plasma were stored in sterile Eppendorf tubes at -80 ºC. The cells were discarded.

Bacteriology processing

A portion of the sputum sample was processed by adding an equal weight of sputolysin (Sputasol; Unipath, Hampshire, UK) and several glass beads (1-1.5 mm in diameter) and incubated for 30minutes at 37 °C during which time they were vortexed for 5-10seconds intermittently. The homogenised samples were diluted 10 fold in brain heart infusion broth and 100 µl aliquots were plated out onto the surface of a range of different media including blood agar, chocolate agar, Mac-Conkey agar, and cysteine lactose electrolyte deficient agar. These plates were incubated for 18 hours at 37 °C in an atmosphere of air + 5% CO_2. After incubation, bacterial colonies were counted and sub-cultured for identification by standard methods. The number of colony forming units/g sputum was calculated from the number of colonies obtained and the dilution of the sputum. The bacteriology results are expressed as the logarithm of the number of colony forming units per millilitre of sputum (log cfu/ml).

Sample storage

All processed serum and sputum supernatants were stored at -80 ºC in our own freezers.

3.3.11 Cytokine measurements

Sputum and serum IL-6 and sputum IL-8 were measured using a quantitative sandwich enzyme immunoassay kit and following the manufacturer's instructions (Quantikine from R&D Systems, Abingdon, UK). The principle of the assay is that a monoclonal antibody specific for IL-6 or IL-8 is pre-coated onto a microplate. Standards, samples and conjugate are pipetted into the wells and any IL-6 or IL-8 present was sandwiched by the immobilized antibody and the enzyme-linked polyclonal antibody specific for IL-6 or IL-8. After a wash to remove any unbound substances and/or antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of IL-6 or IL-8 bound. The colour development was stopped and the intensity of the colour was measured by determining the optical density of each well using a microplate reader set at 540nm the appropriate wavelength for the measured cytokines. A standard curve was created with the standards used with each assay. The level of cytokine present in the samples was determined from the standard curve.

The minimum detectable level of IL-8 was 10 pg/ml and for IL-6 it was 0.7 pg/ml. For all the sputum samples, the concentrations of cytokine are expressed as pg/ml and represent a 10-fold dilution by weight of the original sample.

CRP was measured using a luminometric reader in the hospital laboratory. The minimum detectable level of serum CRP was 0.3 mg/l.

All the samples from one patient were sampled in the same assay/run to avoid inter-assay/run variability.

3.3.12 Statistical analysis

Data were analysed using SPSS version 11 for Windows, STATA-5 software (Stata Corporation, Texas, US). The Kolmogorov-Smirnov test of normality was applied. Normally distributed data were expressed as means with standard deviation (SD), skewed data as medians and inter-quartile ranges (IQR). Skewed data were log 10 transformed to obtain a normal distribution. Comparisons between baseline and exacerbation data, and between the different exacerbation visits for symptoms, lung function parameters, airway and systemic inflammatory markers, were analysed using paired t test, unpaired t test and Mann-Whitney U tests as appropriate.

Spearman's correlation was used to assess the relationships between changes in airway inflammation and recovery time, between serum CRP at day 14 and the time to the next exacerbation. Chi-Square test was used to compare the exacerbation symptoms at the index and recurrent exacerbation. The non log10 transformed values were used to assess the percentage changes between baseline and the different exacerbation visits for each parameter. A probability of error of less than or equal to 5% was considered statistically significant.
Dr Gavin Donaldson supervised and helped me with the statistical analysis mentioned above.

The mixed linear model analysis was performed by Dr Hana Muellerova. Mixed linear models with random effects were applied to compare the evolution of the time curve during the course of exacerbation in two groups of patients separated by a binary trait (e.g. frequent and infrequent exacerbators). First, we examined the best fit values for the curve shape, represented by a time variable, separately for each group. The best fitting curve model (linear, quadratic or cubic) in interaction with the binary trait was then placed in a final model to explain the response of a log 10-transformed biomarker (e.g. serum IL-6, serum CRP). Finally, we produced a visual output from this modelling using the transformed, predicted values for each time point, accompanied by standard error (SE). This part of analysis was supported using the SAS version 8.

3.3.13 Ethics

This study was approved by the East London and City Health Authority Ethics Committee and all patients gave written informed consent.

3.4 Results

3.4.1 Baseline characteristics of the patients

Seventy three exacerbation time courses were collected from 73 different patients; their baseline characteristics are presented in **Table 10**.

Table 10: Baseline characteristics of the 73 COPD patients

	Mean	SD
Age (years)	69.3	**7.8**
Male (%)	54.8	
FEV_1 (litres)	1.08	**0.47**
% Predicted FEV_1 (%)	45	**18**
FVC (liters)	2.36	**0.87**
BMI	26.2	**6.26**
pH	7.41	**0.03**
PaCO2 (kPa)	5.74	**0.95**
PaO_2 (kPa)	8.86	**1.08**
Smoking (pack years) **Active smokers (%)**	48.1 29.4	**34.9**
SGRQ total	59.0	**18.3**
SGRQ symptoms	75.1	**20.1**
SGRQ activity	48.3	**20.6**
SGRQ impact	63.8	**20.7**

In the stable state, 38.3% of the patients reported daily (chronic) dyspnoea, 64.3 % were daily sputum producers, 23.6% reported a daily wheeze and 59.7% reported daily cough.

Disease severity in the stable state was associated with differences in airway and systemic inflammatory markers as reported in **Table 11**.

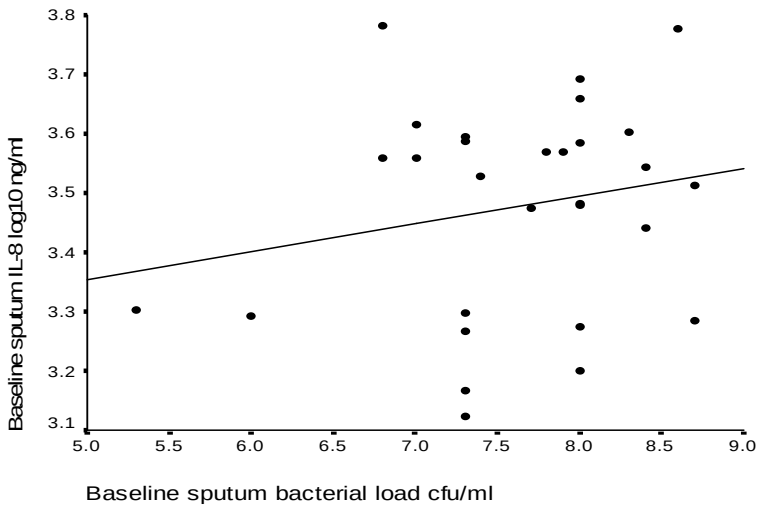
Table 11: Baseline median (IQR) airway and serum inflammatory markers and sputum bacterial load according to GOLD stage of COPD severity

	GOLD I-II disease, n=28	**GOLD III-IV disease, n=45**	**p=**
Sputum IL-8 pg/ml	2177 (1066-3088)	3381 (1978-3930)	**<0.01**
Sputum IL-6 pg/ml	66(32-127)	140 (50-279)	0.22
Serum IL-6 pg/ml	5.5(2.5-9.2)	6.0 (3.1-8.4)	0.30
Serum CRP mg/l	3.9 (2.7-8.2)	7.8(4.9-13.4)	**0.01**
Sputum bacterial load CFU/ml	$10^{7.9(7.35-8.00)}$	$10^{7.8(7.30-8.00)}$	0.96

Patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III and IV disease had a higher median (IQR) CRP than those with GOLD stage I and II disease [7.8 (4.9-13.4) mg/l versus 3.9(2.7-8.2) mg/l, p=0.01]. They also had significantly higher median (IQR) sputum IL-8 than those with GOLD stage I and II disease [3381 (1978-3930) versus 2177 (1066-3088) pg/ml, p=0.004].

When all 73 patients were analysed together, the baseline concentration of sputum IL-8 was related to the baseline sputum bacterial load, r=0.28 and p=0.04 as seen in **Figure 4**.

Figure 4: Relationship between baseline sputum bacterial load and baseline sputum IL-8



The median (IQR) exacerbation frequency was 3.1 (2.0-4.2) per year in this study. Patients with frequent exacerbations had a median (IQR) of 4.2 (3.6-5.7) exacerbations per year compared to 1.9 (1.5-2.3) exacerbations per year for the patients with infrequent exacerbations. There was no significant difference in the baseline characteristics between frequent and infrequent exacerbators, except for the exacerbation frequency itself.

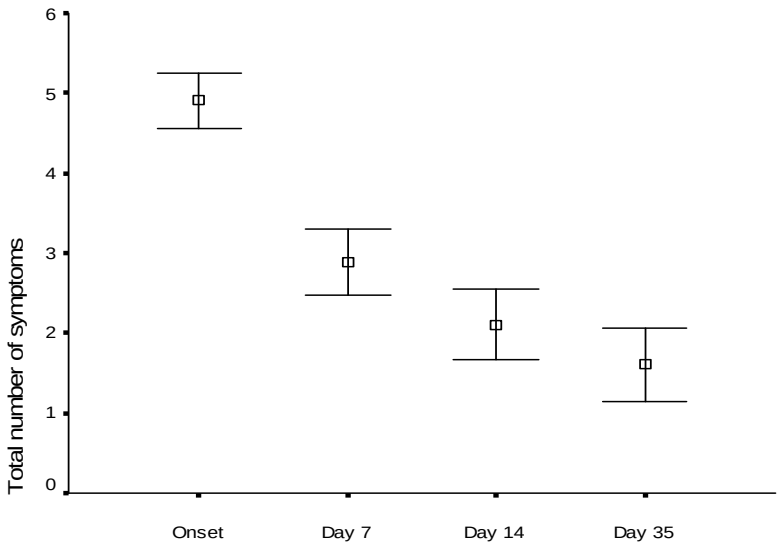
3.4.2 Exacerbation characteristics

At exacerbation patients were seen and sampled on average (SD) 2.3(2.1) days after the onset of the exacerbation and before they started additional treatment. The onset of the exacerbation was determined from the diary cards.

Time course symptoms

Patients had a median (IQR) total symptom score of 5(4-6) at exacerbation as shown in **Figure 5.**

Figure 5: Time course of symptoms at exacerbation and during the recovery



No exacerbation was diagnosed solely on the presence of one major symptom and the presence of a common cold.

The levels of serum IL-6 during the recovery were related to the total symptom score. However there was no significant correlation between the total symptom score at exacerbation and serum IL-6. There was no statistically significant correlation between the levels of sputum IL-6 and the total symptom score at exacerbation or at any of the recovery visits as illustrated in **Table 12**.

Table 12: Relationship between total symptom score at exacerbation and during the recovery and the levels of serum and sputum IL-6.

	Serum		**Sputum**	
	rho=	p=	rho=	p=
Onset	0.23	0.08	0.01	0.92
Day 7	0.28	**0.02**	-0.08	0.54
Day 14	0.33	**0.01**	-0.01	0.90
Day 35	0.34	**0.01**	0.12	0.42

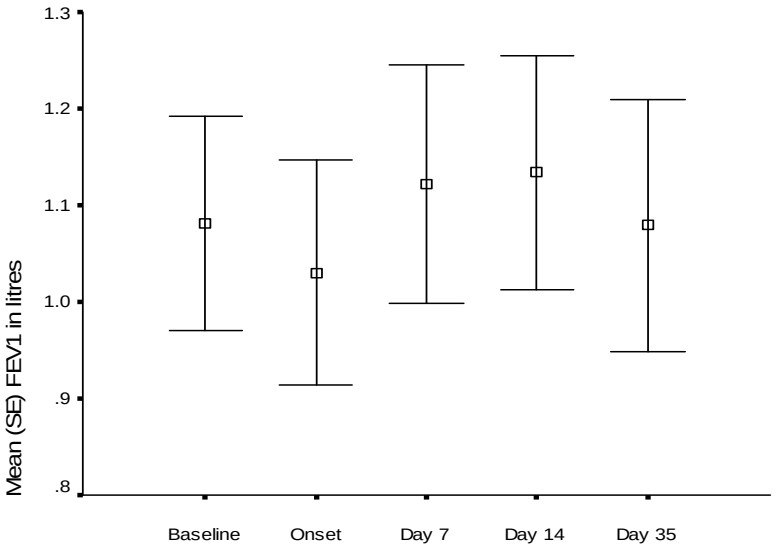
Exacerbation treatment

All exacerbations were treated according to the clinical severity of the episode as deemed necessary by the attending physician. Exacerbation treatment included increased bronchodilaotrs, antibiotics and or oral steroids, 96% (n= 70) of the patients were treated with antibiotics, 67% (n= 50) were treated with oral corticosteroids and 62% (n= 45) were treated with both antibiotics and oral cortico-steroids at exacerbation. At day 14, 36% of the patients were prescribed a second course of oral cortico-steroids.

Time course lung function

The mean (SE) percentage change in FEV_1 at exacerbation compared to baseline was -5.05(-15.02 to + 6.18) %. There was a trend towards a drop in FEV_1 (p=0.07) between baseline and onset of the exacerbation. The time course of FEV_1 is illustrated in **Figure 6.**

Figure 6: Time course of mean (SE) FEV_1 in litres

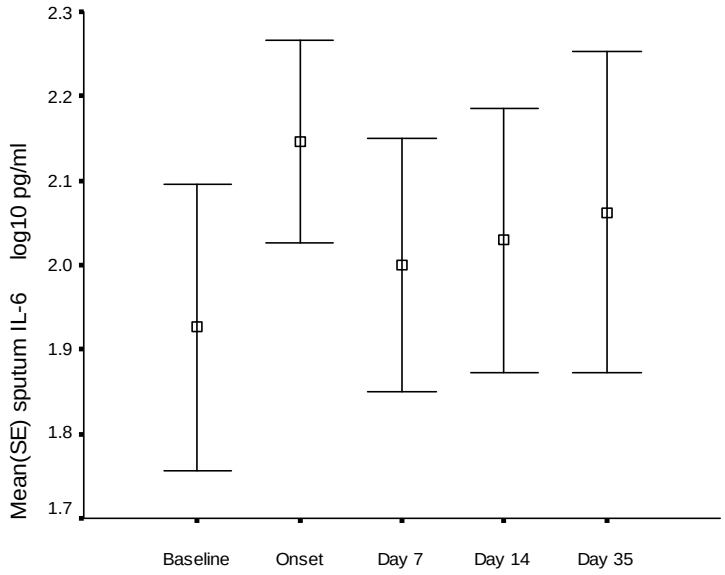


Time course of airway inflammatory markers

There was a significant rise in sputum IL-6 between baseline and onset of the exacerbation, p= 0.02, followed by a return towards baseline by day 7. The changes

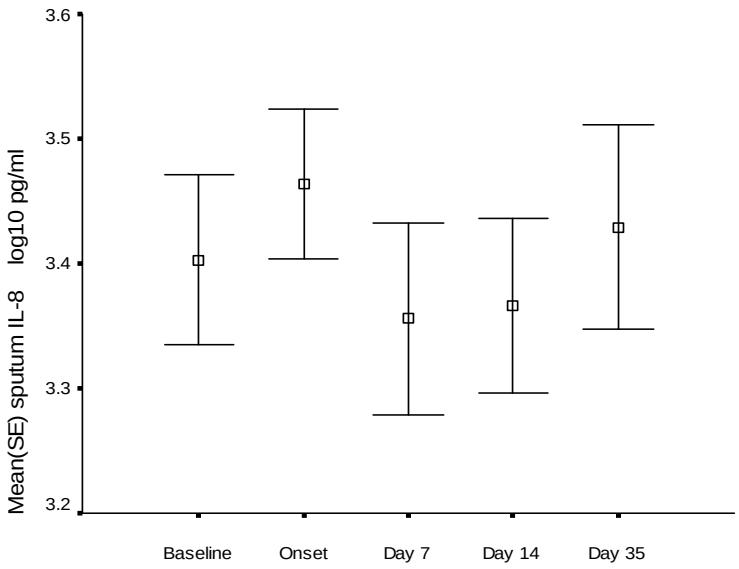
between onset and day 7, 14 and 35 were not statistically significant. The time course of sputum IL-6 is illustrated in **Figure 7.**

Figure 7: Time course of mean (SE) sputum IL-6 in log_{10} pg/ml



There was no statistically significant change in sputum IL-8 between baseline and exacerbation onset. However there was a significant drop between onset of the exacerbation and day seven, from 3263(2249-4140) to 2677 (1633-3897), p= 0.004. The time course of sputum IL-8 is illustrated in **Figure 8**.

Figure 8: Time course of mean (SE) sputum IL-8 in log_{10} pg/ml



Time course of systemic inflammatory markers

There was a significant rise in serum IL-6 between baseline and onset of the exacerbation from 5.74 (3.08-8.69) ng/ml to 12.1 (3.9-24) ng/ml, p=0.001, followed by a drop below baseline to 3.84(1.6-6.4) ng/ml, p=0.001, after seven days. Serum IL-6 then returned towards baseline by day 35. The drop in serum IL-6 between onset of the exacerbation and day seven and between day seven and day 14 were both highly significant (p<0.002). The time course of serum IL-6 is illustrated in **Figure 9.**

There was a significant rise in serum CRP between baseline and exacerbation, from 6.5 (3.5-11.8) mg/l to 12.1(5.8-34.8) mg/l, p=0.001. By day 7 serum CRP had returned towards baseline. The drop in serum CRP between onset of the exacerbation and day seven was highly significant (<0.01 when comparing drop from onset). The time course of serum CRP is illustrated in **Figure 10.**

Figure 9: Time course of mean (SE) serum IL-6 in log_{10} pg/ml

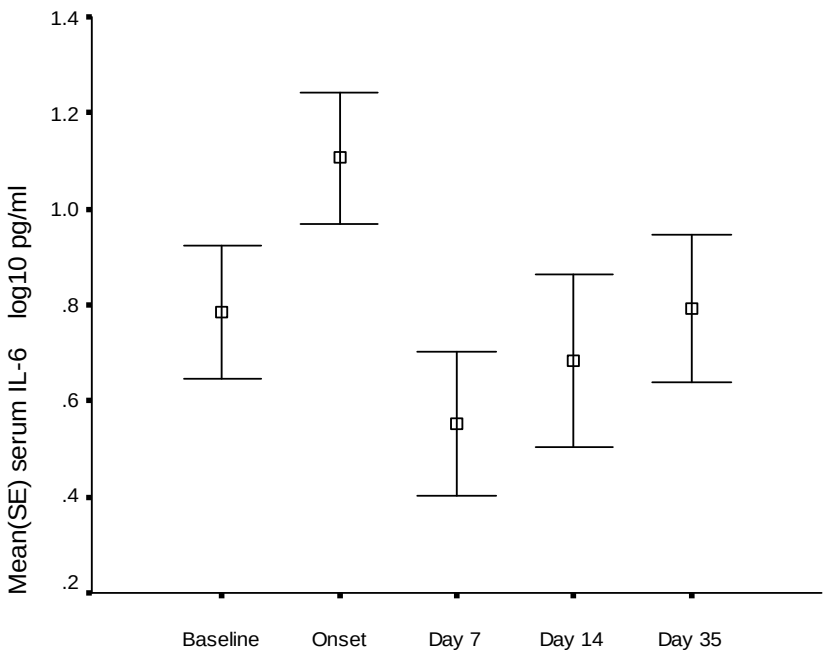
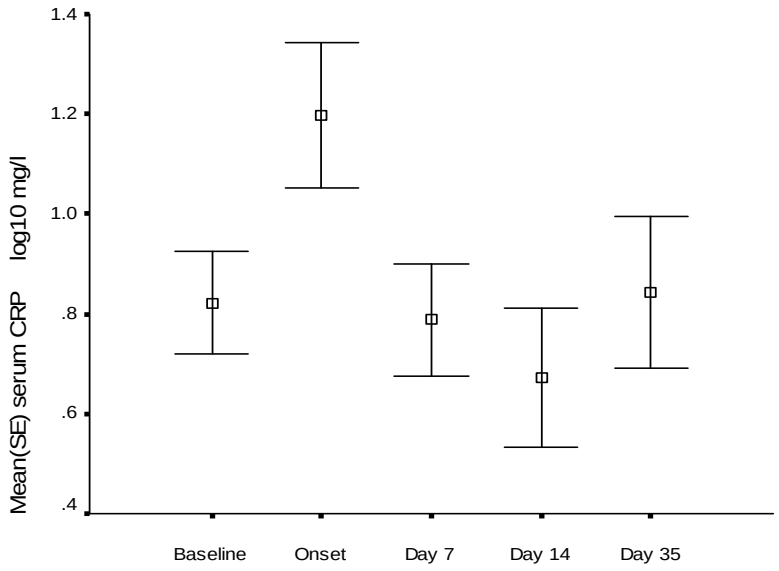


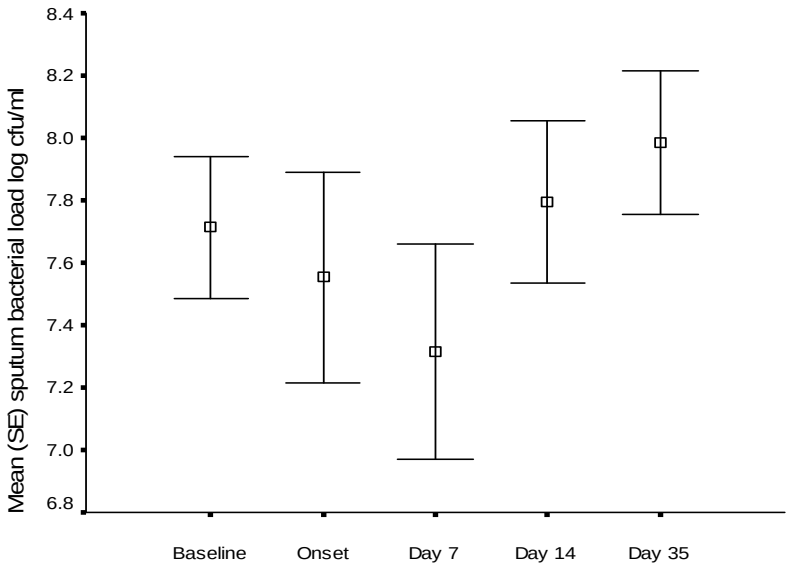
Figure 10: Time course of mean (SE) serum CRP in log_{10} mg/l



Time course of sputum bacterial load

There was no statistically significant change in the sputum bacterial load between the stable state and exacerbation when all the patients were assessed together, p=0.83. However, sputum bacterial load at day seven was significantly lower than in the stable state and compared to exacerbation onset, p=0.01 and p=0.02. The time course of the sputum bacterial load is illustrated in **Table 11**.

Figure 11: Time course of mean (SE) sputum bacterial load in log CFU/ml



Between day 7 and day 35 there was a significant increase in the lower airway total bacterial load, p=0.002. Most patients (97%) received a course of antibiotics between exacerbation onset and day 7. There was a trend towards a higher sputum bacterial load at day 35 compared to baseline, p=0.08.

3.4.3 Outcome of exacerbations treated with and without oral steroids

Our patients were treated with antibiotics and/or oral steroids according to the severity of the exacerbation as judged by the attending physician. The repeated sampling during the recovery period gave us a unique opportunity to assess the impact of treatment with oral steroids on airway and systemic inflammation during the recovery.

Patients with GOLD stage III and IV disease had steroids more often than those with GOLD I and II disease, 76% versus 52%, Chi square 4.26 and p=0.03. However, the total number of symptoms experienced by GOLD stage III and IV patients who received steroids at onset was not significantly different from patients with the same severity who did not receive steroids.

There was no difference in sputum IL-6 in the stable state between patients treated with or without steroids at onset of exacerbation. However, serum IL-6 was higher at baseline in those treated with steroids compared to those treated without steroids at onset and after 14 days (p=0.003).

Baseline FEV_1 was lower in patients treated with oral steroids at onset of the exacerbation, p=0.004. Patients treated with a course of oral steroids at onset had a lower FEV_1 and a higher percentage drop in FEV1 compared to baseline (p=0.03). There was no significant difference in the total symptom score, in sputum IL-6, serum CRP and IL-6 between those treated with or without steroids at onset of the exacerbation.

At day 14, patients who received a further course of oral steroids (36% of patients) had higher symptom scores, had significantly higher levels of sputum IL-6 (p=0.04) and higher levels of serum IL-6 (p=0.01), as illustrated in **Table 13**.

Table 13: Differences in symptoms, FEV_1, sputum and serum IL-6 in patients treated with or without steroids at exacerbation.

	Baseline			**Exacerbation onset**			**At day fourteen**		
	Steroid	No steroid	p	Steroid	No steroid	p	Steroid	No steroid	**p**
Median(IQR) number of symptoms				5(4-6)	4(4-6)	0.68	3(2-4)	1(0-3)	**0.01**
Mean (SD) FEV_1 l	1.00(0.37)	1.22(0.62)	0.06	0.91(0.36)	1.25(0.60)	**0.004**	1.15(0.58)	1.11(0.38)	0.79
Mean(SD) log_{10} sputum IL-6 pg/ml	1.84(0.67)	2.08(0.36)	0.18	2.10(0.53)	2.21(0.35)	0.39	2.21(0.39)	1.88(0.55)	**0.04**
Mean (SD) log_{10} serum IL-6 pg/ml	0.82(0.43)	0.60(0.26)	**0.04**	1.08(0.53)	0.97(0.45)	0.43	0.86(0.48)	0.54(0.34)	**0.01**
Mean (SD) log10 serum CRP mg/l	0.85(0.27)	0.70(0.43)	0.17	1.18(0.61)	1.20(0.45)	0.92	0.74(0.55)	0.61(0.40)	0.36

3.4.4 Relationship between severity of disease and exacerbation severity

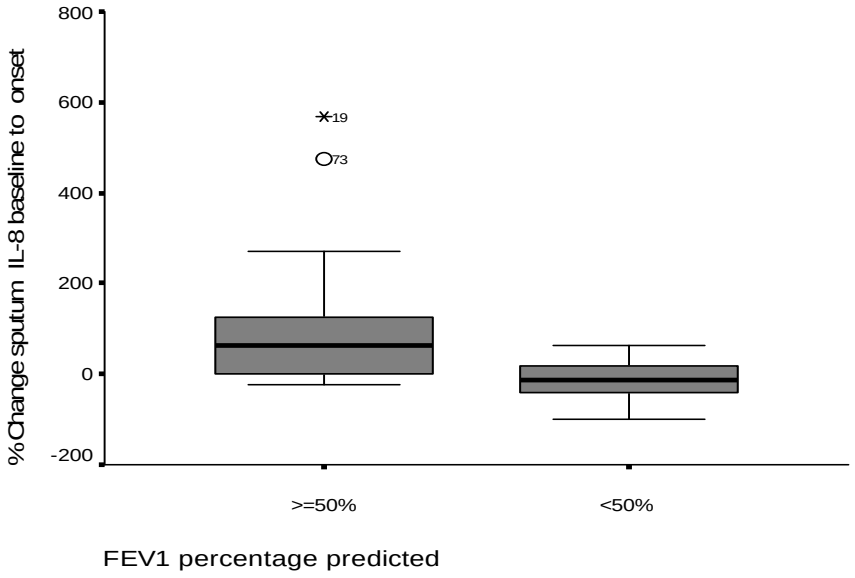
The total symptom score at each of the exacerbation visits was compared between patients with %FEV_1 below and above 50% using a Mann-Whitney U test and is represented in **Table 14**. There was no significant difference in the total symptom scores at exacerbation between the two groups. Patients with GOLD III-IV disease showed a trend towards a higher total symptom score during the recovery period at day 7 and 14 after the exacerbation.

Table 14: Time course of symptom score according to GOLD stage of COPD

	Median (IQR) total symptom score in patient with GOLD III-IV	Median (IQR) total symptom score in patient with GOLD I-II	p=
Onset	5(4-6)	5(4-6)	0.96
Day 7	3(2-4)	2(1-3)	0.06
Day 14	3(1-3)	1(1-0)	0.05
Day 35	1(0-3)	0	0.11

There was no statistically significant difference in the percentage drop in FEV_1 at exacerbation between patients with GOLD III-IV compared to patients with GOLD I-II disease, p=0.15. Patients with GOLD III-IV had a median (IQR) percentage change FEV_1 between baseline and onset of 2.94(-14.28 to 8.06) compared to 6.75(-17.97 to 1.61) in patients with GOLD I-II disease. Patients with GOLD III-IV COPD had a smaller percentage change in sputum IL-8 at exacerbation compared to patients with GOLD I-II disease, p<0.01.

Figure 12: Percentage change in sputum IL-8 levels according to GOLD stage of disease



3.4.5 Relationship between in airway and systemic inflammatory markers, bacterial load and recovery

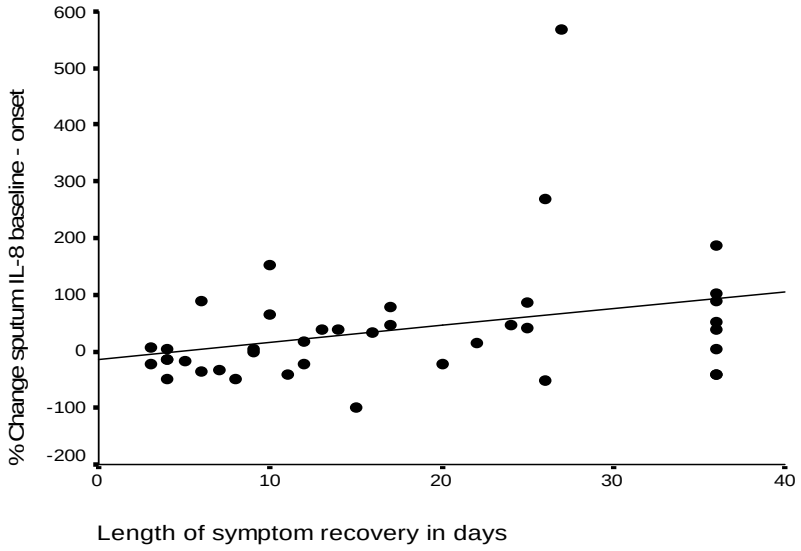
The exacerbation recovery data for symptoms and PEFR were obtained from the daily diary cards. The median (IQR) symptom recovery time was 9 (4-18) days and the median (IQR) PEFR recovery time was 5 (1-11) days. In 11% of the cases PEFR did not recover by day 35.

In 60% (n=44) of the patients, symptoms had recovered to baseline by day 35. In 23% (n=17) of the cases, symptoms did not recover to baseline by day 35, and in the remaining 16% (n=12) of the cases, diary card data did not allow us to establish an exact recovery date

The percentage change in sputum IL-8 between baseline and exacerbation was directly related to the symptom recovery time, rho=0.41 and p=0.01 in a univariate analysis. In a multivariate analysis, the percentage change in sputum IL-8 was related

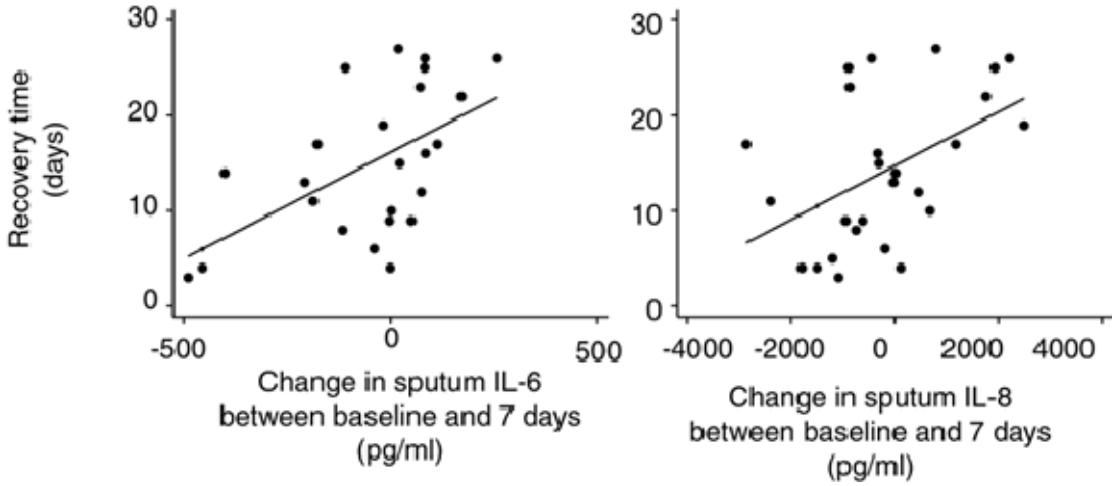
to the recovery time independent of the severity of disease and the exacerbation frequency, p=0.03.

Figure 12: Relationship between symptom recovery and percentage change in sputum IL-8 between baseline and onset of exacerbation.



There was a significant relationship between the symptom recovery time and the changes in sputum IL-6 and IL-8 between baseline and day 7; (rho=0.57 and p=0.004 for sputum IL-6, rho=0.48 and p=0.01 for sputum IL-8).

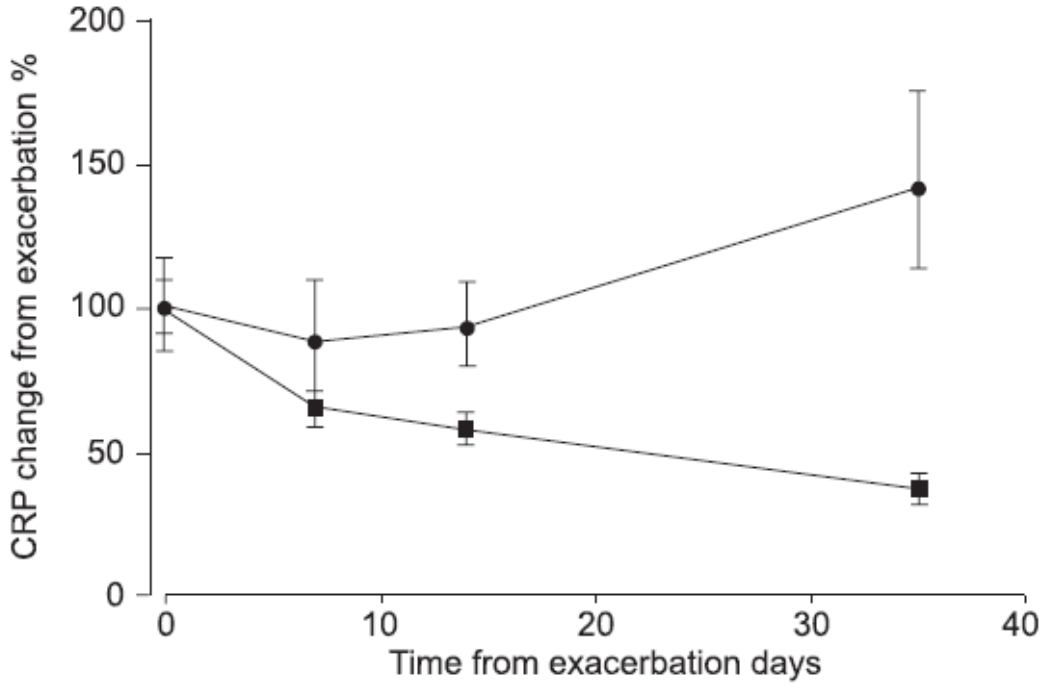
Figure 14: Relationship between recovery time and change in sputum IL-6 and IL-8 between baseline and day 7.



However the symptom recovery time was not related to the changes in sputum IL-6 and serum IL-6 between baseline and exacerbation onset or any other time point during the recovery.

In a mixed linear model analysis, patients with non-recovered exacerbations at day 35 had a persistently higher serum CRP (but not IL-6) concentration during the recovery period, compared with those patients who had recovered (p=0.03).
This is illustrated in **Figure 15.**

Figure 15: Time trend of serum C-reactive protein (CRP) in patients whose symptoms had recovered (●) and those whose symptoms had not recovered (■)



On this graph the estimates from the mixed linear model analysis on log transformed data were anti-logged. The data are presented as means (SEM) expressed as a percentage of the value of the exacerbation onset sample (time=0).

There was no statistically significant relationship between the change in bacterial load between baseline and exacerbation and the symptom recovery time.

3.4.6 Colds and symptom recovery

Patients presenting with symptoms of a common cold at COPD exacerbation are known to have a prolonged recovery time and higher airway inflammation at onset (Seemungal et al 2000, Bhowmik et al 2000). However the evolution of the airway inflammation over time has not been described. In our study patients presenting with symptoms of a common cold at exacerbation onset had significantly higher levels of sputum IL-6 than patients without a cold, mean (SD) sputum IL-6 at onset was 2.24(0.43) versus 1.99(0.52) $\log_{10}$ pg/ml, p=0.04. Seven days after onset these patients still had significantly higher levels of sputum IL-6 than patients presenting without a cold at onset, mean (SD) sputum IL-6 at day 7 was 2.16 (0.48) versus 1.70(0.58) $\log_{10}$ pg/ml, p=0.003. In a multivariate analysis, this relationship between a cold at exacerbation onset and persistently high sputum IL-6 after 7 days remained significant (p=0.016), independent of $\%FEV_1$, active smoking, exacerbation frequency, sputum bacterial load in the stable state and at exacerbation onset.

3.4.7 Differences in the systemic and airway inflammatory changes in patients with frequent and infrequent exacerbations

We compared the percentage changes between baseline and exacerbation for sputum IL-6, sputum IL-8, serum CRP and serum IL-6 between frequent and infrequent exacerbators and did not find any statistically significant differences among the two groups as illustrated in **Table 15.**

77.8% (28/36) of patients with frequent exacerbations were treated with oral steroids compared to 55% (20/36) patients with infrequent exacerbations (Chi-squared p=0.046).

There was no significant difference in the recovery time between frequent and infrequent exacerbators (p=0.76).

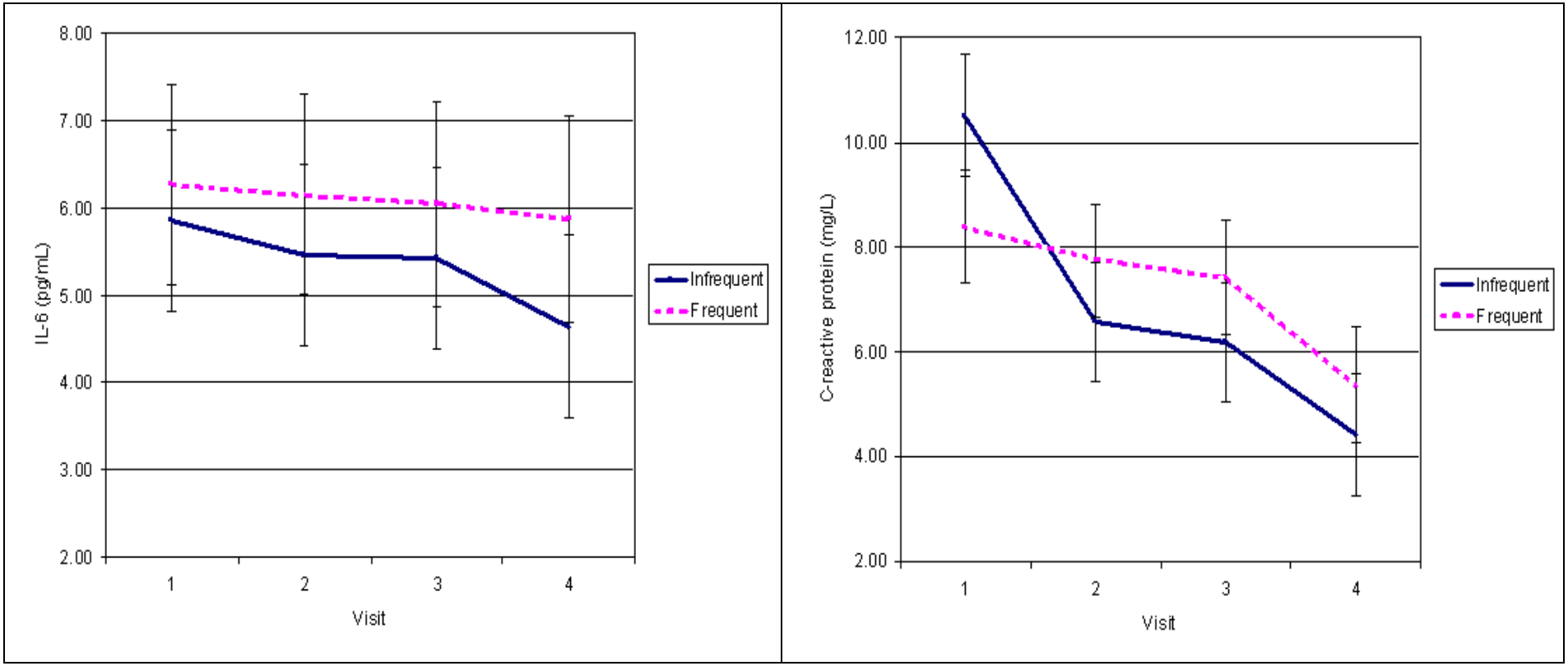
Table 15: Difference in airway and systemic inflammatory changes between baseline and exacerbation in frequent and infrequent exacerbators

	Infrequent exacerbators	Frequent exacerbators	p=
% Change Sputum IL-6	76(-48 to 385)	-7.00(-49 to 160)	**0.41**
% Change Sputum IL-8	39(-21 to 89)	0.82(-39 to 19)	**0.06**
% Change Serum IL-6	68(9 to 439)	50(-33 to 268.00)	**0.53**
% Change Serum CRP	42(-7 to 78)	35.00(-4 to 68)	**0.92**

We then evaluated the difference in systemic inflammation between frequent and infrequent exacerbators using a mixed linear model analysis. There was a significant difference in the time trends of serum IL-6 and serum CRP between exacerbation onset and day 35 in frequent and infrequent exacerbators, $p<0.05$ for both. Frequent exacerbators had a smaller reduction in systemic inflammation between exacerbation and day 35, despite treatment, compared to infrequent exacerbators. This is illustrated in **Figure 16.**

There was too much variability in the airway markers to analyse them using the mixed linear model analysis.

Figure 16: Differences in serum IL-6 and CRP between frequent and infrequent exacerbators with the mixed linear model.



3.4.8 Recurrent exacerbations

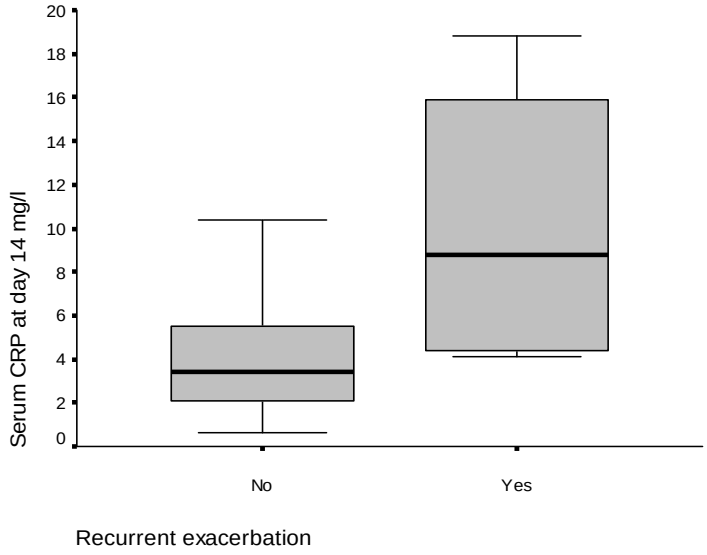
Recurrent exacerbations occurring close to each other are a frequent cause of readmission to hospital. We compared the airway and systemic inflammation during the initial sampled exacerbation in patients with and without another exacerbation (=recurrent exacerbation) within 50 days. We were able to determine the number of patients with a recurrent exacerbation within 50 days of the index exacerbation from the diary card data.

Sixteen patients (22%) had a recurrent exacerbation within 50 days of the index exacerbation. These recurrent exacerbations were all treated exacerbations. Patients who presented with symptoms of a common cold at the first exacerbation were likely to present with a cold at the recurrent exacerbation (χ^2=6.11 and p=0.01). However this relationship was not statistically significant for any of the other exacerbation symptoms. The interval between the index exacerbation and the recurrent exacerbation was not related to the presence of a cold at the index exacerbation (p=0.67) or at the recurrent exacerbation (p=0.82).

Patients with a recurrent exacerbation within 50 days of the index exacerbation had higher concentrations of serum CRP at day 14 than those without a recurrent exacerbation, CRP was 8.8 (4.4-15.9) mg/l versus 3.4 (2.0-7.7) mg/l, p= 0.007. This is illustrated in **Figure 17**. In a multivariate analysis, the CRP at day 14 was related to a recurrent exacerbation within 50 days independent of the disease severity, exacerbation frequency and treatment of the index exacerbation with oral steroids (p=0.004). No such relationships were demonstrated for the other markers and time-points

There was no significant relationship between recurrent exacerbations and the concentrations of airway and systemic inflammation in the stable state prior to the index exacerbation. The severity of the index exacerbation was not related to recurrent exacerbations occurring in the fifty days afterwards. Total symptom score for non-recurrent versus recurrent exacerbations was 3 (2.0–3.7) versus 3 (2–4), respectively (p=0.99), and percentage change FEV_1 was -4.8% (-11.6– -1.2%) versus -12.9% (-14.1– -6.6%), p=0.20).

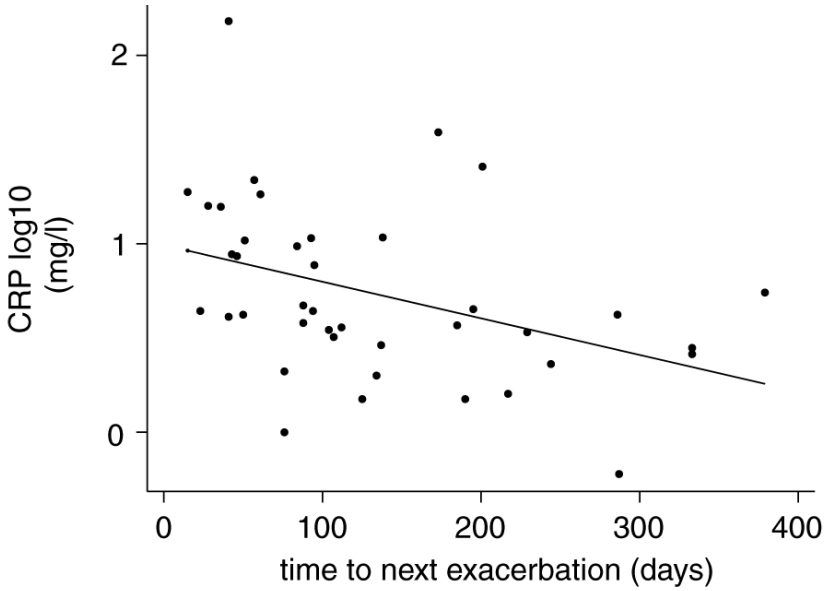
Figure 17: Levels of serum CRP at day 14 in patients with and without a recurrent exacerbation within 50 days.



3.4.9 Time to the next exacerbation

Diary card data on the time to the next exacerbation were available for 61/73 patients. The median (IQR) time to the next exacerbation was 93(50-178) days. Seventy five per cent of the subsequent exacerbations were treated exacerbations. There was a significant relationship between the level of serum CRP at day 14 and the time to the next exacerbation, (rho= -0.47 and $p<0.01$) such as a higher CRP fourteen days after the index exacerbation was associated with a shorter time to the next exacerbation. This relationship is illustrated in **Figure 18.**

Figure 18: Relationship between serum CRP at day 14 and time to the next exacerbation.



3.4.10 Exacerbations requiring hospital admission

Nine out of the seventy three sampled patients were admitted to hospital within the 35 days following the onset of their exacerbations. One patient required admission to intensive care and invasive mechanical ventilation. No patients died during the study period.

The nine patients had a significantly higher median (IQR) change in FEV_1 at exacerbation onset compared to the 64 patients treated in the community, -16(-11 to -34) ml versus -4(-14 to +7) ml change in FEV_1, p=0.02. Hospitalised patients also had a significantly higher level of serum IL-6 at the exacerbation visit compared to patients treated in the community, mean(SD) serum IL-6 was 1.45(0.59) log_{10} ng/ml versus 0.99(0.46) log10 ng/ml, p=0.01. There was no significant difference in the number of people living alone between the two groups. There was no significant difference in the baseline FEV_1, sputum and serum IL-6 level, and the baseline and exacerbation sputum IL-8 levels between the 2 groups.

3.5 Discussion

This is the first study to evaluate prospectively the time course of symptoms, airway and systemic inflammatory markers in the stable state, at exacerbation and during the recovery in a cohort of well characterised patients with COPD. We studied the differences at exacerbation in total symptom score, airway inflammation and airway bacterial load in patients with mild to moderate COPD compared to patients with severe to very severe COPD. We assessed the relationship between severity of COPD and symptom and PEFR recovery time, and the need for antibiotics and oral steroids. We determined the relationship between the presence of a common cold at exacerbation onset and the persistence of heightened airway inflammation after 7 days. We also investigated the relationships between airway and systemic inflammatory markers, and lower airway bacterial load at exacerbations of COPD and the symptom recovery time. We assessed the differences in the inflammatory changes and bacterial load changes at exacerbation in patients who are frequent or infrequent exacerbators.

We describe, for the first time, a relationship between the level of CRP 14 days after an exacerbation and the time to the next exacerbation. We also demonstrate a way to identify patients at increased risk of a recurrent exacerbation within 50 days of the index exacerbation.

All patients recruited into this study completed daily diary cards for changes in exacerbation symptoms and reported exacerbations to the study team as soon as possible after the onset. A unique feature of using daily diary cards in our cohort study is that we can collect objective data on the start and end of an exacerbation and accurately determine non-recovery of symptoms and PEFR. In addition, the study design allowed sampling early in the time course of the exacerbation when the inflammatory markers probably reached their initial peak. Most of the baseline samples were obtained prior to the index exacerbation and at least six weeks away from the last exacerbation. All the exacerbation samples were taken before starting treatment with antibiotics and/or oral corticosteroids.

In the stable state

COPD is associated with increases in airway inflammation, and IL-8 is one of the markers of the neutrophilic inflammation found in this condition. IL-8 levels are related to the degree of airflow obstruction (Yamamoto et al 1997), smoking status (Hill et al 2000 b) and bacterial colonization (Soler et al 1999, Hill et al 2000 a). In agreement with previous studies, the present data showed the level of sputum IL-8 in the stable state is related to the severity of the airway obstruction and the lower airway bacterial load.

Epidemiological studies showed a direct relationship between systemic inflammation and the severity of the airway obstruction. The present data showed higher levels of serum CRP in patients with severe to very severe COPD compared to patients with mild to moderate COPD.

At exacerbation

This is the first study to describe prospectively the time course of airway and systemic inflammatory markers in the stable state, at exacerbation and during the recovery. As our patients were followed up with daily diary cards, we could establish precisely the time between onset of symptoms and sampling. This is a unique feature of our study. The previous time course studies started from the day the patient presented to the GP/hospital, the interval between onset of symptoms and sampling was variable. In most studies the stable state samples were taken after the exacerbation, there were no prospective baseline, as in our study. The changes in airway and systemic inflammation we describe are in agreement with the previous time course studies. From our data it appears that systemic inflammatory markers give a more accurate picture and more information regarding the exacerbation than airway inflammatory markers.

During recovery

We have previously described that the recovery time of an exacerbation is related to the severity of the exacerbation as measured by symptom scores and peak flow changes (Seemungal et al 2000), and that patients with a longer exacerbation recovery times are more likely to have had a delay between exacerbation onset and

treatment initiation (Wilkinson et al 2004). Our previous work also described that a significant number of COPD exacerbations do not recover to baseline after six weeks (Seemungal et al 2000).

An important finding from the present study is that the intensity of the change in sputum IL-8 at exacerbation was directly related to the symptom recovery time. We describe for the first time a direct relationship between the severity of the airway inflammation at exacerbation and symptom recovery time. We also show that those patients who, despite treatment with antibiotics and steroids, have persistently high levels of sputum IL-6 and IL-8 at day seven compared to baseline have a prolonged symptom recovery time. Our group had previously described that exacerbations with symptoms of a common cold at onset were associated with a longer recovery time and higher levels of sputum IL-6 at onset (Seemungal et al 2000, Bhowmik et al 2000). In the present study we demonstrate that the longer symptom recovery time is due to persistence of increased airway inflammation after 7 days.

For the first time, we have identified a direct relationship between non-recovery of symptoms 35 days after an exacerbation and persistently of high levels of serum CRP during the recovery period. Our results thus suggest that the systemic inflammatory response is an important marker of the recovery time and outcome of the exacerbation.

Exacerbation frequency

It is now recognised that exacerbation frequency is an important outcome in COPD, as patients prone to frequent exacerbations have impaired health status (Seemungal et al 1998), reduced physical activity status (Donaldson et al 2005 a), increased lower airway bacterial colonisation (Patel et al 2002) and a faster lung function decline (Kanner et al 2001, Donaldson et al 2002, Dowson et al 2001, Donaldson et al 2005 b). These frequent exacerbators also have increased airway inflammatory markers in the stable state (Bhowmik et al 2000) and we have previously reported that frequent exacerbators have a faster rise in systemic inflammation over time compared to infrequent exacerbators (Donaldson 2005 b). In the present study we establish that patients who are frequent exacerbators have significantly higher levels of serum IL-6 and CRP during the recovery period, and this may account for the increased

morbidity and mortality seen in this patient group at exacerbation, as they are also the patients prone to more frequent hospital admissions (Garcia-Aymerich et al 2003, Soler-Cataluna et al 2005).

We have previously described that the increase in airway and systemic inflammation in stable COPD patients, over time, is, directly linked to disease progression (Donaldson et al 2005 b) and thus it can be extrapolated that this delayed recovery in inflammatory markers in the frequent exacerbators may be one of the mechanisms for the faster FEV_1 decline and disease progression observed in this group (Kanner et al 2001, Donaldson et al 2002, Donaldson et al 2005 b). The slower resolution of inflammatory markers at exacerbation in the frequent exacerbator group may also lead to the increased airway inflammation observed in the stable state (Bhowmik et al 2000). In this study we did not see a difference in recovery time between frequent and infrequent exacerbators, and this is consistent with previous findings from our group (Seemungal et al 2000).

Exacerbations impose a considerable burden on health care services. Although most COPD exacerbations are treated in the community, they are an important cause of hospitalization; and are responsible for around 10% of all acute medical admissions (Donaldson et al 2006). Within the year after admission to hospital with an exacerbation, 63% of the patients are readmitted at least once (Roberts et al 2002), and this figure increased to 79% in patients admitted with acute hypercapnic respiratory failure during the first admission (Chu et al 2004). These repeat admissions were mainly due to recurrent exacerbations and known risk factors for readmission included being a frequent exacerbator and having more severe COPD (Garcia-Aymerish et al 2003). Another study described how frequent severe COPD exacerbations requiring repeated hospital admissions (3 or more) in the year following a index admission were associated with a greater mortality compared to patients without readmissions (Soler-Cataluna et al 2005). The authors also found that those recurrent hospital admissions with exacerbations constituted an independent risk factor for mortality. Identification of patients likely to have a recurrent exacerbation early in the course of their index exacerbation may allow implementation of appropriate preventive strategies.

Recurrent exacerbations and time to the next exacerbation

A further unique finding of this study is the important association between the raised serum CRP during the recovery period and recurrent exacerbations. COPD patients with an elevated (above 8.8 mg/l) level of CRP 14 days after the index exacerbation are more likely to have a recurrent exacerbation within 50 days. These finding needs to be explored further with interventional studies of therapies such as oral anti-inflammatory agents targeted at those patients with a high CRP after exacerbation. Interventions used in this manner may be specifically capable of preventing recurrent exacerbations. Reducing recurrent exacerbations would lead to substantial reductions in morbidity, mortality and health care costs associated with COPD.

We have also previously described that early presentation to health care professionals can affect the outcome of the exacerbation and reduce hospitalization (Wilkinson et al 2004). The present study emphasises further the importance of following up COPD exacerbations and this issue could be incorporated into COPD management guidelines, especially routine follow up at 14 days after the index exacerbation. CRP can be easily and rapidly measured in many health care settings and can therefore be integrated into the routine follow-up of patients with COPD exacerbations without major additional costs.

A high CRP level despite treatment with antibiotics and steroids could be related to several factors such as failure to eradicate the causative agent or resistance of the responsible organism to the treatment prescribed. It may also be possible that the heightened inflammatory status, and/or the modification of the bacterial flora colonizing the lower airways after the initial index exacerbation, facilitate infection by a different organism (Sethi et al 2004). Changes in the strains of bacteria colonizing the lower airways have also been implicated in COPD exacerbations and strain changes may be involved in exacerbation recurrence (Sethi et al 2002).

Comparing the nature of the index and recurrent exacerbation, we found that only cold symptoms were significantly associated with both events. This suggest that patients who develop recurrent exacerbations may be more susceptible to respiratory viral infections and this is consistent with another study from our group where

frequent exacerbators were more likely to acquire colds than infrequent exacerbators (Hurst et al 2005). Further studies are required to evaluate the origin of the inflammatory response at a recurrent exacerbation.

3.6 Summary

In conclusion we have demonstrated that:

- The intensity of rise in sputum IL-8 at exacerbation onset is related to the recovery time.

- The total symptom score is related to the levels of serum IL-6 during the recovery at day 7, 14 and 35.

- The response to treatment is related to recovery time as persistently high airway inflammation at day 7 is related to a prolongd recovery time.

- Non recovery is associated with persistently heightened systemic inflammation.

- Patients with a cold at exacerbation have a prolonged recovery due to persistently high airway inflammation.

- 22% of the patients had a recurrent exacerbation within 50 days of the index exacerbation.

- Patients who had a recurrent exacerbation had a higher level of serum CRP at day 14 than those who did not.

- The level of serum CRP measured 14 days after the index exacerbation was directly related to the time to the next exacerbation.

- Frequent exacerbators have a reduced response to therapy, which results in persistently higher systemic inflammatory markers and which may explain the greater decline in lung function observed in these patients.

- Serum inflammatory markers were better predictors of non-recovery and recurrent exacerbations than sputum inflammatory markers.

- Hospital admissions were associated with more severe exacerbations in terms of FEV_1 drop and serum IL-6 rise.