Airway inflammation in asthma : from concept to the clinic

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Chapter
3

Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma

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Abstract

Background Airway hyperresponsiveness, induced sputum eosinophils and exhaled nitric oxide (NO) have all been proposed as non-invasive markers to monitor airway inflammation in patients with asthma. The aim of the present study was to compare the changes in each of these markers as obtained by inhaled glucocorticoids in one single study.

Methods In a randomized, double-blind, placebo-controlled, parallel study, 25 patients with mild asthma (19-34 yr, FEV_1>75% predicted, PC_{20}<4 mg/ml) inhaled fluticasone propionate (500 µg, bid) for 4 weeks. PC_{20} to histamine, sputum eosinophils and exhaled NO were determined at weeks 0, 2, 4, and after 2 weeks wash-out (week 6). Sputum was induced by inhalation of hypertonic (4.5%) saline, and eosinophils counts were expressed as % non-squamous cells. Exhaled NO (ppb) was measured by chemiluminescense.

Results Within the steroid group, there was a significant increase in PC_{20}, decrease in sputum eosinophils and decrease in exhaled NO as compared with baseline at weeks 2 and 4 of treatment ($p<0.01$). Subsequently, each of these variables showed significant worsening during two weeks run-out as compared with week 4 ($p<0.01$). These changes were significantly different from those in the placebo group ($p<0.05$), except for the changes in sputum eosinophils from baseline to week 2 and from week 4 to 2 weeks wash-out. There were no significant correlations between the changes in the three markers in either group at any time.

Conclusion We conclude that 4 weeks of treatment with inhaled steroids leads to improvements in airway hyperresponsiveness to histamine, eosinophil counts in induced sputum, and exhaled nitric oxide levels. Our results suggest that these markers may provide complementary information when monitoring anti-inflammatory treatment in asthma.

Introduction

Asthma is an inflammatory disease of the airways, associated with airway hyperresponsiveness to various bronchoconstrictor stimuli, such as histamine (1). The accompanying inflammation is characterized by the presence of inflammatory cells, such as T-lymphocytes, neutrophils and eosinophils, and their cytokines in the airway mucosa as demonstrated in bronchial biopsy specimens (2,3). The current treatment of asthmatic patients is based on reducing or preventing airway inflammation as guided by lung function and symptoms (1,4). To monitor airway inflammation more closely, measurement of non-invasive and sensitive markers of inflammation, such as airway hyperresponsiveness (5), sputum eosinophils (6) or exhaled NO (7) during treatment follow-up in patients with asthma has recently been suggested.

To date, inhaled glucocorticoids are the most effective treatment of asthma not only reducing symptoms and airway hyperresponsiveness (8), but also leading to an improvement of airway inflammation (9). However, there is recent evidence that therapy according to the present international guidelines provides only partial suppression of airway inflammation, as shown by persisting eosinophilic inflammation in the bronchial (sub)mucosa after long-term inhaled steroid treatment (5).

Among the non-invasive techniques, hypertonic saline-induced sputum has been shown to be a reliable method to measure eosinophilic airways inflammation (6,10,11). The number of eosinophils in sputum is associated with asthma severity (10) and decreases following inhaled steroid treatment (12). In addition, nitric oxide levels in exhaled air have also been proposed as marker for disease severity in asthma (7,13). Indeed, inhaled glucocorticoids decrease the levels of exhaled NO in patients with asthma (14), in a dose-dependent way (15).

Although the effects of inhaled steroids on sputum eosinophils and exhaled NO have been well established, comparative analysis is required before any of these markers can be recommended in the monitoring of asthma therapy. In the present study we investigated treatment-induced changes in airway hyperresponsiveness, sputum eosinophils and exhaled NO in asthma. To that end, we performed histamine challenge, induced sputum and exhaled NO measurements before, during and after 4 weeks treatment with fluticasone propionate or placebo in steroid-naive patients with asthma.

Methods

Subjects

Twenty-five non-smoking, atopic patients (9 female and 16 male, 19-34 years) with mild persistent asthma (1) voluntered to participate in this study (Table 1). Symptoms of episodic chest tightness and wheezing were treated by on-demand usage of inhaled salbutamol alone, which was discontinued at least 8 h before the measurements. Two

Table 1. Characteristics of the subjects

Subject No.	Sex	Age (yr)	FEV_1 (% pred)	PC_{20} (mg/ml)	EO (%)	NO (ppb)
Steroid						
1	M	24	77	0.07	1.4	5.85
2	M	24	104	0.37	3.8	10.75
3	M	21	104	0.39	5.8	11.81
4	F	23	88	0.55	7.6	5.28
5	F	20	83	0.71	4.0	3.42
6	M	24	103	0.72	0.0	8.24
7	M	23	94	1.29	3.6	10.21
8	F	24	101	1.81	0.2	7.25
9	F	21	99	2.05	NA	2.17
10	M	28	104	2.54	3.4	2.15
11	F	21	98	3.14	1.4	3.92
12	M	27	99	3.14	0.2	4.62
		23.3 (2.4)¶	96.2 (9.0)¶	0.91 (1.62)*	3.40 (0.0,7.6)§	5.57 (2.15,11.81)§
Placebo						
13	M	24	82	0.11	21.2	13.41
14	F	21	108	0.11	0.0	6.57
15	M	29	111	0.14	24.6	12.05
16	M	34	83	0.30	0.0	4.17
17	M	21	98	0.46	1.6	13.40
18	M	24	80	0.54	NA	14.08
19	M	25	86	0.73	0.0	3.26
20	M	24	98	0.77	1.8	4.22
21	F	24	106	0.89	3.2	2.48
22	M	28	90	1.00	1.2	3.36
23	F	28	106	1.20	NA	5.05
24	F	25	98	1.51	0.0	5.82
25	M	19	97	1.70	0.4	9.26
		25.1 (3.9)¶	95.6 (10.6)¶	0.52 (1.38)*	1.20 (0.0,24.6)§	5.82 (2.48,14.08)§

¶ = mean (SD), * = geometric mean (SD) in DD, § = median (range), NA = not applicable

weeks before the study all subjects were free of symptoms of respiratory tract infection. Atopy was indicated by a positive skin prick test (> 3 mm wheal) to one or more of 10 common airborne allergen extracts (Vivodi–agnost, ALK, The Netherlands). The forced expiratory volume in one second (FEV_1) was greater than 75 % of the predicted value (16), and all subjects were hyperresponsive to inhaled histamine (PC_{20} < 4 mg/ml) (17). The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and a signed informed consent was obtained from all volunteers.

Design

The study had a randomized, double-blind, placebo-controlled parallel design. During screening, the selection criteria were checked for all subjects. Before entering the treatment period baseline values of PC_{20} histamine and percentage eosinophils in

induced sputum were determined. These two measurements were carried out on two separate days, with a 2-4 days interval. Prior to histamine challenge and sputum induction, baseline values of FEV_1 and exhaled NO were recorded. This sequence of measurements was used at all time points of the study. Directly following the second baseline visit, the subjects were treated with inhaled fluticasone propionate (500 µg bid) or placebo for a period of four weeks. The measurement of PC_{20} histamine, sputum eosinophils, FEV_1 and exhaled NO were repeated during the treatment period (at weeks 2 and 4) and during wash-out at two weeks after the treatment period.

Histamine challenge

Histamine challenges were performed according to a standardized methodology (17). Histamine-di-phosphate (Sigma Chemicals, St.Louis, MO, USA) in PBS was stored at 4°C and administered at room temperature. Doubling concentrations between 0.06 and 16 mg/ml were used. The aerosols were generated by a DeVilbiss 646 nebulizer (output: 0.13 ml/min), connected to an in- and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T). Each dose was inhaled through the mouth by tidal breathing for 2 minutes at 5-minute intervals, with the nose clipped (17). The airway responses to the inhaled aerosols were measured using FEV_1, recorded by a dry rolling-seal spirometer (Morgan Spiroflow, Morgan UK) and monitored on-line by a personal computer with a special soft-ware program. Before each test FEV_1 was measured in triplicate, for calculation of mean baseline levels (17). The airway response was recorded at 30 and 90 seconds after each dose. After each inhalation, the lowest, technically satisfactory FEV_1 value was applied in the analysis to calculate the percentage fall in FEV_1 from baseline. The test was discontinued if FEV_1 was decreased by 20% or more. The provocative concentration causing 20 % fall in FEV_1 (PC_{20}) was calculated by log-linear interpolation of the final two data points.

Sputum induction

Sputum was induced and processed by the so called full-sample method (18) according to a protocol that has been validated in our laboratory (6). Hypertonic saline aerosols (NaCl 4.5%) were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer with a calibrated particle size (MMAD 4.5 mm) at maximal output (2.5 ml/min). The aerosols were administered to the subjects through a 100 cm long tube with an internal diameter of 22 cm, and inhaled via the mouth through a two-way valve (No. 2700; Hans-Rudolph, Kansas City, MO, USA), with the nose clipped. Before inhalation of the aerosols, baseline FEV_1 was recorded and, for safety reasons, 400 µg salbutamol was administered through a metered dose inhaler (Volumatic). Subsequently, the subjects inhaled hypertonic saline aerosols during 2 x 5 min and 1 x 10 min intervals. After each inhalation, or as soon as the subjects experienced cough, they were asked to blow their nose, to rinse their mouth and throat with water, and to expectorate sputum into a clean plastic container by coughing. After testing, FEV_1 was measured, and salbutamol was administered if needed.

Sputum processing and cell differential counts

The volume of the induced sputum samples was determined and mixed with an equal volume of 0.1% sputolysin (dithiotreitol, Calbiochem, USA) (6). To ensure complete homogenization, the samples were placed in a shaking water bath at 37 °C for 15 minutes, once interrupted by gently mixing the sample. The homogenized sputum was centrifuged (350 x g) for 10 minutes at room temperature. The cell pellet was resuspended in PBS to a final volume of 2-5 ml, followed by filtration through a gauze (pore-size approximately 1 mm) to remove clumbs. Total cell counts were performed in a haemacytometer (Tamson, Zoetermeer, The Netherlands). Subsequently the sample was diluted with PBS to a final concentration of $\pm 0.3 \times 10^6$ cells/ml which was used for preparation of the cytocentrifuge slides (1500 rpm, 3 minutes, 50 ml/slide) (Shandon 3, Life Sciences International, Veldhoven, The Netherlands). Differential cells counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells were performed on Diff-Quik stained, cytospins by a qualified cytopathologist. To correct for the variable salivary contamination, differential leukocyte and cylindric epithelial cell counts were expressed as a percentage of 250 nucleated cells excluding squamous cells. For each sample, differential cell counts were performed twice by the same observer, and the mean data were used in the analysis. A sputum sample was considered adequate when the percentage squamous cells was less than 80%. The reproducibility of the sputum cell counts as obtained by this method has been shown to be satisfactory (6). To ensure a blind analysis of the sputum samples, all cytocentrifuge slides were coded before analysis by an investigator who was not involved in the counting.

Exhaled NO

Exhaled NO levels were measured by a chemiluminescense analyzer (Sievers NOA 270B) according to a standardized procedure (7), which has previously been applied by our lab (19). The subjects were connected to a closed system to avoid contamination of the measurements with ambient NO. Pressured air with low NO concentration (< 1ppb) was administered through a 150 L reservoir connected to the inspiratory side of a Hans-Rudolph three-way valve. The subjects performed a slow vital capacity manoeuvre with a constant expiratory flow of 10L/min against an expiratory resistance of 3-4cm H_2O. Expiratory NO concentration was sampled continuously from the centre of the mouthpiece at a sample flow of 440 ml/min, and the average concentration (in parts per billion; ppb) was determined for a period of 10 seconds (7). Baseline values of exhaled NO were obtained from the mean values of the two NO measurements recorded before histamine challenge and sputum induction, because the reproducibility was good (intraclass correlation coefficient, $R_i > 0.92$).

Analysis of data

PC_{20} was log-transformed before statistical analysis, and expressed as geometric mean (SD) in doubling dose. To test for differences between and within the treatment groups in general, multivariate analysis of variance (MANOVA) was applied for FEV_1 and log PC_{20}, whilst Kruskal-Wallis test was used for sputum eosinophils and exhaled NO. The changes in PC_{20} (expressed in doubling doses: DD) within each treatment group were

analysed using Student's paired t-test, whilst the changes in PC_{20} between both groups were tested using Student's unpaired t-test. Since exhaled NO levels and sputum eosinophils were not normally distributed, these markers were analysed non-parametrically. The Wilcoxon signed-rank test was used to test for the differences within each treatment group. Furthermore, the Mann-Whitney signed-rank test was applied to test for the differences between the groups in changes in sputum eosinophils and exhaled NO at all time-points as compared with baseline. Finally, Spearman rank correlation analysis was used to examine the relationship between the changes in PC_{20}, sputum eosinophils and exhaled NO. Results were considered significant if p value < 0.05. All statistical analyses were performed using SPSS.

Results

Three of the subjects dropped out during the run-out period due to a history of respiratory tract infection (#5 and #6), or because of taking an anti-histamine (#11). Three subjects (#9, #18 and #23) did not produce adequate sputum at baseline, whilst subject 21 and 7 were not able to produce sputum at week 2 and week 4, respectively These time points were handled as missing data.

Lung function and histamine challenge

At baseline there were no significant differences in FEV_1 and PC_{20} between the groups (p>0.19; table 1). During the study there were no significant changes in FEV_1 in the two groups (p>0.96, MANOVA). In the placebo group there were no significant changes in PC_{20} (p = 0.92, MANOVA) while in the steroid treated group PC_{20} increased significantly at week 4 compared with baseline values (mean change 2.01 (95% CI 0.683 to 2.090); p = 0.001; fig 1). After a two week washout period PC_{20} decreased again compared with week 4 by –1.75 (–1.831 to –0.582) doubling doses (p = 0.002; table 2, fig 1). These changes were significantly different from the changes in the placebo group (p<0.003; table 3).

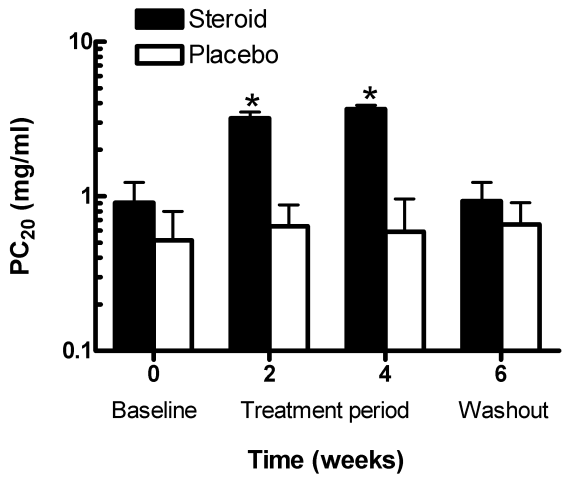

Figure 1. Airway hyperresponsiveness to histamine (PC_{20}) at baseline, at 2 and 4 weeks of treatment, and after 2 weeks wash-out in steroid (closed bars) and placebo group (open bars). shown as geometric mean doubling dose. * = significant difference between the two groups.

Table 2. Airway hyperresponsiveness, sputum eosinophils and exhaled NO during and after steroid and placebo treatment

	Baseline	Week 2	Week 4	Run-out
Steroid group				
PC_{20} (mg/ml)	0.91 (1.62)	3.19 (1.54)¶	3.67 (1.05)¶	0.93 (1.50)§
Eosinophils (%)	3.40 (0.00,7.60)	0.30 (0.00,3.00)¶	0.20 (0.00,1.60)¶	4.41 (1.40,20.00)§
NO (ppb)	5.57 (2.15,11.81)	1.54 (0.11,4,86)¶	1.48 (0.59,3.68)¶	3.50 (0.90,12.89)§
Placebo group				
PC_{20} (mg/ml)	0.52 (1.38)	0.64 (1.21)	0.59 (1.86)	0.66 (1.26)
Eosinophils (%)	1.20 (0.00,24.60)	1.20 (0.00,18.56)	3.47 (0.00,16.60)	3.75 (0.60,30.00)
NO (ppb)	5.82 (2.48,14.08)	5.03 (0.59,18.73)	5.26 (0.17,11.38)	5.36 (1.94,21.28)

Values of PC_{20} expressed as geometric mean (SD) in DD, values of sputum eosinophils and exhaled NO expressed as median (range), ¶p<0.01 as compared to baseline, §p<0.01 as compared to week 4

Sputum eosinophils

The mean (SD) percentage of squamous cells in this study was 33.4 (17.6)%. Baseline sputum eosinophils were not significantly different in the two groups (p = 0.31; table 1). There were no significant changes in sputum eosinophils within the placebo group (p = 0.85, MANOVA), but in the steroid treated group a significant decrease in sputum eosinophils was observed compared with baseline values (mean change at week 4 –2.46 (95% CI –4.260 to –0.660)%; p = 0.01) with a subsequent worsening in the washout period compared with week 4 (mean change 6.13 (95% CI 0.804 to 11.459)%; p = 0.03; table 2, fig 2). The changes in sputum eosinophils were not significantly different between the two groups when baseline values were compared with week 4, or week 4 values were compared with those in the washout period (table 3).

Exhaled NO

At baseline exhaled NO levels were not significantly different in the two groups (p = 0.55; table 1). During the study there were no significant changes in exhaled NO levels

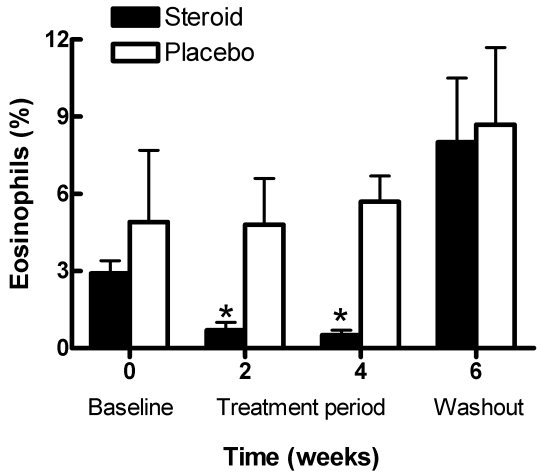

Figure 2. Mean eosinophil counts in induced sputum at baseline, at weeks 2 and 4 of treatment, and after 2 weeks of wash-out in steroid treated (closed bars) and placebo groups (open bars). * = significant difference between the two groups.

Table 3. Comparison of change in airway hyperresponsiveness, sputum eosinophils and exhaled NO between steroid and placebo treatment

	Baseline – Week 2	Baseline – Week 4	Week 4 – Run-out
Δ PC_{20} (mg/ml)			
Steroid	1.80 (1.38)	2.01 (1.60)	-1.75 (1.17)
Placebo	0.32 (0.59)	0.19 (0.97)	0.17 (1.15)
p-value	0.004	0.003	0.001
Δ Sputum eosinophils (%)			
Steroid	–1.40 (-7.60,0.40)	-1.90 (-7.60,0.00)	2.81 (1.40,18.80)
Placebo	0.20 (-18.80,9.80)	1.00 (-11.40,11.20)	0.82 (-7.45,20.80)
p-value	0.13	0.03	0.15
Δ Exhaled NO (ppb)			
Steroid	-3.81 (-10.10,-1.09)	-3.89 (-9.90,-0,75)	2.12 (0.16,9.21)
Placebo	-0.62 (-2.67,4.65)	-1.71 (-5.15,1.09)	0.40 (-1.21,10.76)
p-value	0.0001	0.007	0.049

Values of changes PC_{20} expressed as geometric mean (SD) in DD, values of changes in sputtum eosinophils and exhaled NO expressed as median (range)

in the placebo group (p = 0.54, MANOVA; table 2) but in the steroid treated group the levels of exhaled NO decreased significantly at week 4 compared with baseline values with a mean change of –4.88 (95% CI –6.862 to –2.892) ppb (p < 0.001), with a subsequent increase during the washout period compared withweek 4 of 3.65 (95% CI 0.882 to 6.423) ppb (p = 0.016; table 2, figure 3). These changes in exhaled NO levels were significantly different from the changes in the placebo group between baseline and week 4 (p = 0.005; table 3).

Relationship between observed changes

Within the steroid group there were no significant correlations between the changes in PC_{20}, sputum eosinophils and exhaled NO at any time point (Pearson's r< 0.56, p>0.15; figures 4-6).

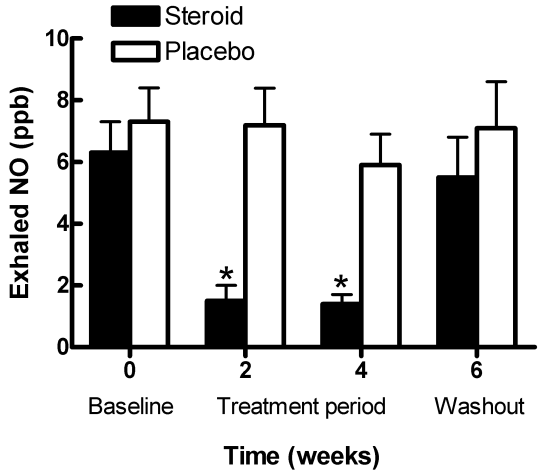

Figure 3. Mean levels of exhaled nitric oxide (NO) at baseline, at weeks 2 and 4 of treatment, and after 2 weeks of wash-out in the steroid treated (closed bars) and placebo groups (open bars). * = significant difference between the two groups.

Discussion

The results of this study indicate 4 weeks of therapy by inhaled steroids lead to improvements in airway hyperresponsiveness, sputum eosinophils, and levels of exhaled NO in patients with mild atopic asthma. In addition, it appears that the improvement in these markers are lost 2 weeks after cessation of treatment. This suggests that each of these markers is useful for monitoring patients with asthma, even though there might be small differences between the markers in the earliest response to anti-inflammatory treatment.

To our knowledge this is the first study comparing the treatment-induced changes in airway hyperresponsiveness to histamine, eosinophils counts in induced sputum, and exhaled NO in a group of asthmatic patients. Our study confirms and extends the results of others who have demonstrated the beneficial effect of glucocorticoids on each of these markers separately. In accordance with Kraan *et al.*, we showed an improvement of 2 doubling doses in airway hyperresponsiveness after 4 weeks of treatment with inhaled

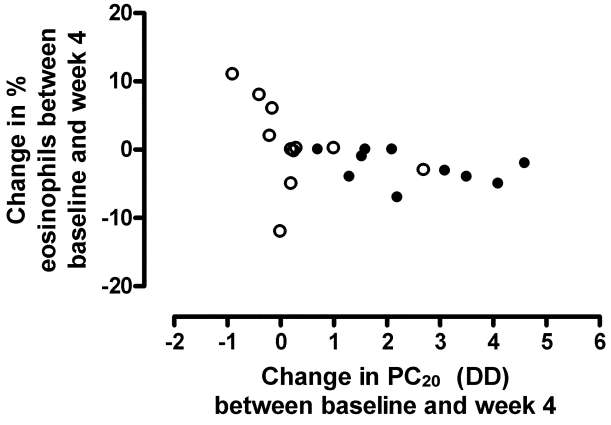

Figure 4. Relationship between the change in sputum eosinophils and the change in PC_{20} histamine at week 4 compared with baseline (closed circles = steroid group; open circles = placebo group).

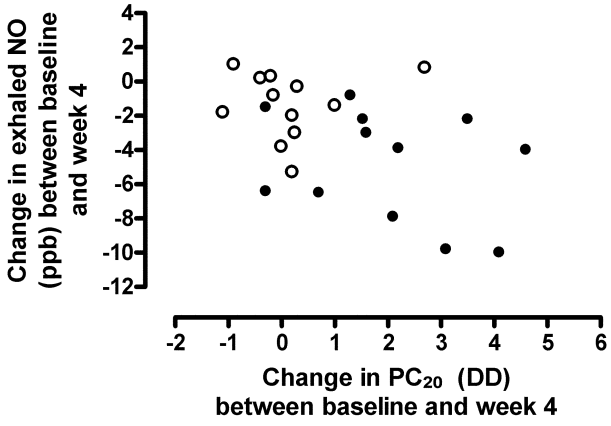

Figure 5. Relationship between the change in exhaled NO levels and the change in PC_{20} histamine at week 4 compared with baseline (closed circles = steroid group; open circles = placebo group).

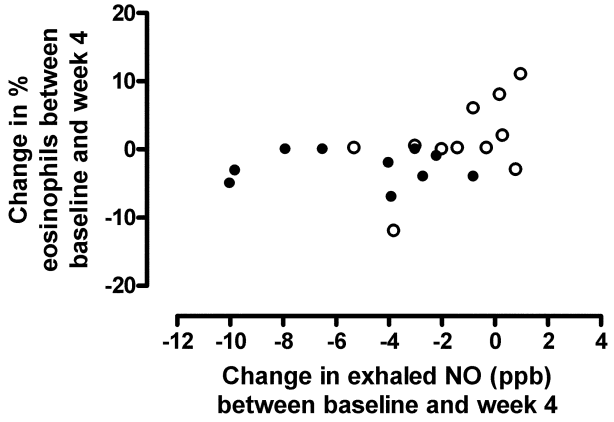

Figure 6. Relationship between the change in sputum eosinophils numbers and the change in exhaled NO levels at week 4 compared with baseline (closed circles = steroid group; open circles = placebo group).

steroids (20). Furthermore, our findings are in agreement with those of Keatings *et al.* (12) and Kharitonov *et al.* (14), who demonstrated a decrease in sputum eosinophils and exhaled NO, respectively, after inhaled steroid treatment.

Although cross-sectional relationships between airway hyperresponsiveness, sputum eosinophils and exhaled NO in asthma have been previously reported (10,21), there are only limited data on the comparison of within-subject changes in these markers during treatment follow-up. Our results are in agreement with those of Baraldi *et al.* who also failed to demonstrate a correlation between steroid-induced changes in PD_{20} and sputum eosinophils (22). The absence of such relationships may reflect the partially distinct pathophysiological backgrounds of these markers, and might be indicative of possible independent, complementary clinical information during anti-inflammatory therapy.

We do not believe that our data were influenced by measurement errors, since we used validated and reproducible methods (6,7,17,19). All subjects in this study were carefully selected to be non-smokers with stable, atopic asthma, who had not used inhaled steroids for at least 1 month prior to the study. We had chosen a relatively high dose of inhaled steroids as the present intervention in order to ensure an optimal anti-inflammatory effect. To avoid carry-over effects, the histamine challenge for determination of PC_{20} and the sputum induction were separated by 2-4 days. Furthermore, exhaled NO levels at these two days appeared to be highly reproducible. Our inability to demonstrate significant improvement in lung function following steroid treatment may be due to the normal baseline levels of FEV_1 in our study (77-111% of the predicted value).

How can the present findings be interpreted? First, glucocorticoids are likely to decrease the percentage sputum eosinophils by reducing the release and effects of cytokines like interleukin-5 (IL-5) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on eosinophil infiltration and survival (23-25). Second, the steroid-induced reduction in exhaled NO can be explained by the inhibition of inducible NO synthase (iNOS) expression directly and/or indirectly by reduction in the levels of stimulatory cytokines,

for instance in epithelial cells (26). Finally, the improvement in the physiological marker, PC_{20}, is likely to be due to effects of steroids on the presence and activity of multiple (infiltrative and resident) cells (5,8,9,27). Hence, it may not be surprising that the steroid-induced changes in the three markers were not significantly correlated to each other. Apparently, the earliest improvements of eosinophils in response to steroid treatment is somewhat out of phase as compared to the other two markers. However we believe that this has little implications, given the consistency in the changes between the markers after 4 weeks of treatment.

What are the clinical implications of the present findings? Treatment according to the current international guidelines is based on minimising symptoms and optimising lung function (1). However, frequently, this fails to provide complete suppression of airway inflammation (5). It has been postulated that persistent airway inflammation in asthma leads to airway remodelling and an irreversible loss of lung function (28,29). This may require the use of more direct markers for monitoring airway inflammation (10,30). Indeed, a recent study by Sont *et al.* demonstrated that the adjustment of long-term inhaled steroid treatment, additionally guided by the level of airway hyperresponsiveness, leads to a significantly better clinical, as well as histological, outcome as compared to treatment based on symptoms and lung function alone (31). Based on the present data, it needs now to be addressed in long-term prospective trials as to whether monitoring sputum eosinophils and/or exhaled NO can provide similar benefits in asthma management.

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Chapter 3