

The relationship between airway hyper-responsiveness, markers of inflammation and lung function depends on the duration of the asthmatic disease

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Summary

Background The combination of airway hyper-responsiveness, eosinophilic airway inflammation, and lung function impairment is considered as a hallmark of bronchial asthma. Since airway function might change with time in chronic asthma, the association between parameters which are characteristic of asthma could be different in subjects with different durations of the disease.

Objective We assessed whether in patients with asthma the relationship between airway hyper-responsiveness, non-invasive markers of airway inflammation, and baseline lung function depended on the duration of the disease.

Methods Sixty-six non-smoking patients with mild to moderate allergic asthma without corticosteroids were assigned to two groups, according to a duration of the disease (time interval since doctor's diagnosis) of either ≤ 16 years (median 8 years; mean FEV₁, 92.6% pred.; $n = 34$) or > 16 year (median 25 years; mean FEV₁, 87.9% pred.; $n = 32$).

Results Groups did not differ statistically in PC₂₀FEV₁ of methacholine, sputum composition, levels of exhaled nitric oxide (NO), lung function parameters, or history of treatment. There were significant correlations between PC₂₀FEV₁, eosinophils and NO in patients with a duration of the disease ≤ 16 year, but no relation to lung function. In contrast, patients with a duration > 16 year showed a correlation between PC₂₀FEV₁ of methacholine and lung function but not eosinophils or NO. In both groups, eosinophils and NO were associated with each other. These results were corroborated by the statistical procedure of factor analysis that revealed 'inflammation' and 'lung function' as major entities and found 'responsiveness' to be associated with only one of them in each group.

Conclusion Our data demonstrate that with a shorter duration of the asthmatic disease airway hyper-responsiveness is associated with airway inflammation, whereas with a longer duration it is associated with impaired lung function, suggesting that in chronic asthma ongoing alterations become the primary determinant of functional characteristics.

Keywords airway hyper-responsiveness, inflammation, lung function

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Introduction

The assessment of non-specific airway hyper-responsiveness is widely used to characterize patients with asthma and to quantify the effects of anti-asthmatic therapy [1–5]. In asthma, hyper-responsiveness is accompanied by eosinophilic airway inflammation, as detectable through bronchial mucosal biopsies [6] and induced sputum [7]. Accordingly, a relationship between airway hyper-responsiveness and sputum eosinophils or, alternatively, the concentration of exhaled nitric oxide (NO), has been reported [8,9]. In addition, airway responsiveness is known to be related to baseline lung function [10–12]. However these findings are not supported by all studies and some have noted that there were no significant correlations

between airway responsiveness, markers of airway inflammation, and lung function [2,3,13,14].

Irrespective of the potential issues of sample size or differences in methodology, some of the discrepancies might have originated from the fact that groups of patients with different characteristics were studied. In particular, patients might have differed in the duration of their disease, and this difference could have been associated with different patterns of inflammation and functional impairment, according to the concept of airway remodelling in chronic asthma [6]. It seems conceivable to hypothesize that such alterations, accumulated over many years, could also affect the relationship between airway hyper-responsiveness, indices of airway inflammation, and lung function, all of which are obtainable through non-invasive methods.

Based on these considerations we investigated in the present study, whether the relationship between the degree of airway responsiveness to methacholine, to the cellular composition of induced sputum, to the concentration of exhaled NO, and to baseline lung function was depending on the duration of the disease in patients with allergic asthma without corticosteroids.

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Methods

Patients

Sixty-six patients with stable mild to moderate asthma according to the Expert Panel Report II [15] were included in this study. Patients were non-smokers for at least 1 year and none of them had a smoking history of ≤ 10 pack years. Bronchial asthma was diagnosed as episodes of dyspnoea and wheezing accompanied by bronchial hyper-responsiveness to inhaled methacholine, as indicated by a provocative concentration, $PC_{20}FEV_1$, < 8 mg/mL. Predicted values were taken from European Respiratory Society (ERS) recommendations [16] and the duration of the disease was defined as the time interval since the doctor's diagnosis. All patients were treated with short-acting β_2 -adrenoceptor agonists only for at least 8 weeks prior to the tests, were free of respiratory tract infections within 4 weeks, and showed a positive skin prick test to at least one of a set of common allergens. Each patient underwent a methacholine inhalation challenge as well as the measurement of exhaled NO and sputum induction within a time interval of at most 10 days. Written informed consent was obtained from all patients and measurements had been approved by the Ethics Committee of the Medical Board of Schleswig-Holstein.

Lung function and challenge procedures

Lung function was measured following American Thoracic Society (ATS) guidelines [17] and airway responsiveness following a rapid protocol that had been validated previously and shown to yield the same results as the standard Chai-Rosenthal dosimeter method [18]. Provocative concentrations of methacholine ($PC_{20}FEV_1$) causing a 20% reduction in FEV_1 were derived by standard log-linear interpolation.

Measurement of exhaled NO

Following established procedures [19] patients expired against a resistance to assure closure of the velum. After a deep expiration, they performed a rapid inspiration of clean air (< 1 ppb NO) through the mouth with the nose closed, opened their nose again and then expired at a constant rate by maintaining a pressure of 20 mbar over ambient pressure that was displayed for control. Four different flow rates (30–200 mL/s) were achieved by choosing different resistors. Air as sampled between mouthpiece and resistor was led into a nitric oxide analyser (NOA 280, Sievers Instruments Inc., Boulder, CO, USA), whose signals were fed into a computer for determination of NO plateau values. The NO analyser was checked regularly using certified NO calibration gas (Messer-Griesheim GmbH, Krefeld, Germany). Since the level of exhaled NO is flow-dependent and since there were inevitably deviations (up to 10% [19]), of the actually achieved from the target flow rate of 50 mL/s [19], we further improved the precision of NO values by interpolation at a rate of precisely 50 mL/s.

Sputum induction and processing

Patients received 200 μ g salbutamol from a metered-dose inhaler and then inhaled hypertonic saline from an ultrasonic nebuliser (NE-U12, Omron, Tokyo, Japan) for 3×10 min [20]. Lung function was measured before and every 5 min during the procedure. Samples produced after the first (3%

saline), during and after the second (4%), and during and after the third (5%) inhalation period were processed separately and pooled values were derived afterwards [20]. To produce sputum, patients were asked to rinse their mouth thoroughly, blow their nose, swallow water, and to expectorate the sputum into a Petri dish. Samples were kept at 4 °C and processed during the induction procedure or immediately afterwards.

Under an inverted microscope all adequate plugs of sputum were selected. Plugs were defined as the more dense and viscous parts [20] and considered adequate when their origin from the lower respiratory tract was indicated by the presence of non-squamous cells and low or absent squamous cell contamination. The weight of the plugs was determined and twice their volume of SputolysinTM (6.5 mm dithiothreitol, Calbiochem, Bad Soden, Germany) was added. After incubation at 37 °C for 15 min and repeated mixing, PBS was added, cells were resuspended in PBS/BSA (1%), cell counts were assessed in a haemocytometer, viability was determined by trypan blue exclusion, and a sample of 20 000–40 000 cells was used to prepare cytospins. Differential cell counts expressed as percent of non-squamous cells were determined by two independent observers from 400 to 500 non-squamous cells on coded Giemsa-stained cytospin slides and the average of their values was used for analysis.

Statistical analysis

Data on exhaled NO, percent eosinophils and $PC_{20}FEV_1$ were logarithmically transformed to achieve approximately normal distribution. Correspondingly, geometric mean values and standard deviations (SD) were computed for these parameters; geometric SD was expressed as a factor to be multiplied with the geometric mean value. Arithmetic mean values and standard deviations were derived for all other variables, as their distributions were not significantly different from normal. Comparisons between groups were performed by the unpaired *t*-test. Statistical significance was assumed for $P < 0.05$. Within groups Pearson's linear correlation coefficients were computed that were also used in principal component factor analyses with subsequent standardized Varimax rotation [21,22]. In factor analysis, the correlation between variables is attributed to their common dependence from independent hypothetical entities termed 'factors'. The correlation coefficients between the variables and the factors are called 'factor loadings'. Ideally, each variable has a high loading on one factor, while its loadings on all other factors are low. Thus, in essence, the set of variables is separated into disjoint subsets, the correlation within each subset being due to the underlying common factor. The number of factors necessary is chosen to be as small as possible, but large enough to account for most of the information within the data; it is by convention taken from the number of eigenvalues of the correlation matrix with magnitude = 1 [21,22].

Results

Patients' characteristics

Within the whole study population the median duration of the asthmatic disease was 16 years (range 3–36). This value was chosen to define patients with a shorter (≤ 16 years) vs. a longer (> 16 years) duration of the disease to ensure a similar number of patients in both groups and a comparable power of analysis

(Table 1). Corresponding median values of the duration of the disease were 8 and 25 years, respectively. Groups did not differ statistically with regard to PC₂₀FEV₁, cell differentials in induced sputum, the level of exhaled NO, lung function, or history of corticosteroid treatment. In both of them FEV₁ was significantly ($P < 0.05$ each) reduced as compared to predicted values.

Correlation analysis

Both groups showed a statistically significant ($P = 0.001$ each) correlation between the percentages of eosinophils in induced sputum and the levels of exhaled NO (≤ 16 years: $r = 0.53$; > 16 years: $r = 0.58$; Figs 1a and b). The group with a duration

Table 1. Patients' characteristics

Parameter	Dimension	Duration of the disease	
		≤ 16 years $n = 34$	> 16 years $n = 32$
Age	years	26.4 ± 6.6	38.3 ± 11.0
Sex	f/m	16/18	15/17
FEV ₁	L	3.50 ± 0.74	3.16 ± 0.80
FEV ₁	% pred.*	92.6 ± 15.9	87.9 ± 21.0
FVC	L	4.71 ± 0.80	4.26 ± 0.95
FEV ₁ /FVC	%	74.3 ± 9.5	74.3 ± 9.9
PC ₂₀ FEV ₁ Mch†	mg/mL	$0.40 \div 5.1$	$0.28 \div 3.4$
Eosinophils‡,§	%	$2.8 \div 6.5$	$2.8 \div 4.0$
Neutrophils‡	%	21.3 ± 17.0	27.3 ± 22.2
Macrophages‡	%	65.1 ± 22.4	60.9 ± 21.4
Lymphocytes‡	%	1.4 ± 0.8	1.4 ± 0.9
Exhaled NO†,§	ppb	50.9 ± 2.1	53.1 ± 2.2
Duration of the disease¶	years	7.9 ± 5.0	24.7 ± 4.9
Duration of symptoms	years	8.4 ± 5.3	25.4 ± 4.8
Previous ICS treatment	%	21	22
Time of ICS treatment	years	0.5 ± 1.2	0.5 ± 1.3

Values given are mean values $\pm$ SD.

FEV₁ = forced expiratory volume in one second, FVC = forced vital capacity. ICS = inhaled corticosteroids.

PC₂₀FEV₁ = provocative concentration of methacholine that caused a 20% fall in FEV₁.

*predicted values [22]; †geometric mean values and SD (as a factor, indicated by $\div$); ‡in induced sputum; § at a flow rate of 50 mL/s;

¶doctor's diagnosis.

of the disease ≤ 16 years also showed a significant correlation between PC₂₀FEV₁ and exhaled NO ($r = -0.54$, $P = 0.001$; Fig. 2a) as well as sputum eosinophils ($r = -0.50$, $P = 0.002$). In contrast, in patients with a duration of the disease > 16 years, the corresponding correlation coefficients were not statistically significant ($r = -0.09$ and $r = -0.15$, respectively) (Fig. 2b). There was a strong ($P < 0.001$ each) correlation between the percentages of sputum macrophages and neutrophils in both groups (≤ 16 years: $r = -0.77$; > 16 years: $r = -0.87$). Furthermore, the group with duration ≤ 16 years showed statistically significant ($P < 0.05$) correlations between macrophages and eosinophils ($r = -0.56$) or NO ($r = -0.35$) as well as between lymphocytes and PC₂₀FEV₁ ($r = 0.47$).

The relationship between FEV₁% pred. and PC₂₀FEV₁ was significant ($r = 0.62$, $P < 0.001$) in the group with a duration of the disease > 16 years but not in that with a duration ≤ 16 years ($r = 0.31$; n.s) (Figs 3a and b). Similar values were obtained for the FEV₁/FVC ratio ($r = 0.57$ and 0.28 , respectively). There was a close correlation ($P < 0.001$ each) between FEV₁% pred. and the FEV₁/FVC ratio in both groups (≤ 16 years: $r = 0.77$; > 16 years: $r = 0.69$). All other correlation coefficients were not statistically significant from zero.

In contrast to the correlations between parameters within the two groups, there was no significant relationship between the parameters measured and age or the duration of the disease.

Factor analysis

Within the group that showed a duration of the disease ≤ 16 years, three independent factors emerged (Table 2). One of them was represented by PC₂₀FEV₁, exhaled NO and sputum eosinophils, a second one by FEV₁% pred. and FEV₁/FVC and a third one by sputum macrophages and neutrophils. Lymphocytes showed their highest loading on the factor comprising the eosinophils, but the association was not as clear-cut as in the other variables.

The group with a duration of the disease > 16 years demonstrated the presence of four factors (Table 3). One of them comprised exhaled NO and eosinophils, the second one PC₂₀FEV₁, FEV₁% pred. and FEV₁/FVC, the third one macrophages and neutrophils, and the fourth one only lymphocytes. There was a bimodal distribution of either high or low factor loadings, suggesting a rather consistent association of the variables with the underlying factors.

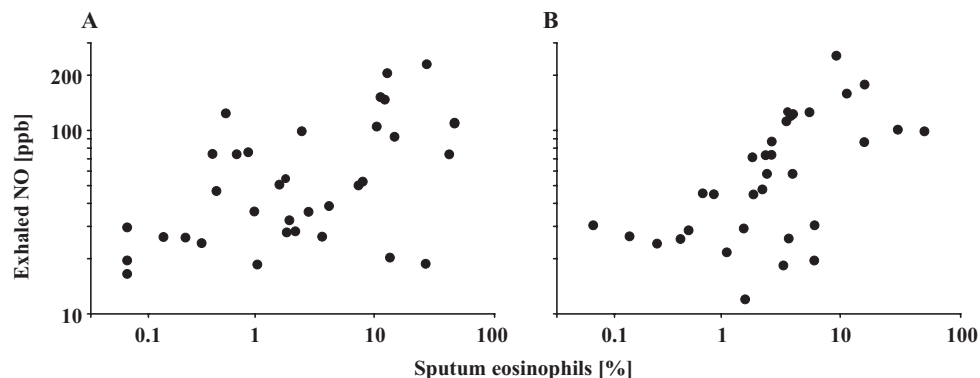


Fig. 1. Relationship between the percentages of sputum eosinophils and the levels of exhaled NO in the two groups of patients with duration of the disease ≤ 16 years (a) and > 16 years (b).

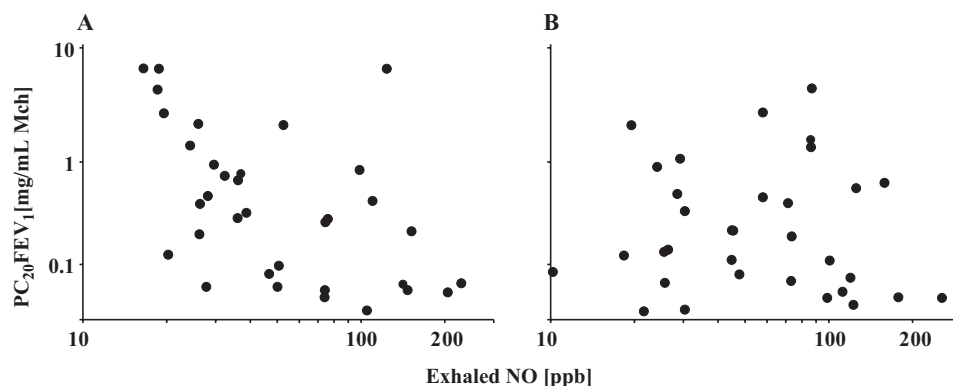


Fig. 2. Relationship between the level of exhaled NO and non-specific airway responsiveness in terms of PC₂₀FEV₁ methacholine in the two groups of patients with duration of the disease ≤ 16 years (a) and > 16 years (b).

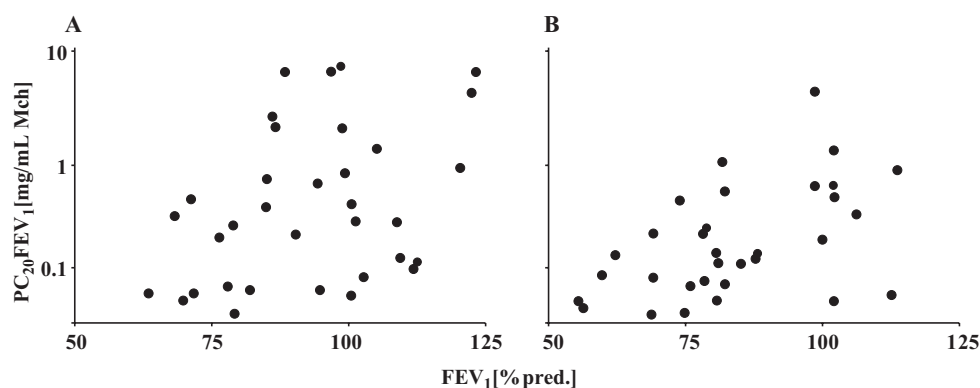


Fig. 3. Relationship between baseline lung function in terms of FEV₁% pred. and non-specific airway responsiveness in terms of PC₂₀FEV₁ methacholine in the two groups of patients with duration of the disease ≤ 16 years (a) and > 16 years (b).

Table 2. Factor loadings in the group of patients showing a duration of asthma ≤ 16 years

	Factor		
	1	2	3
PC ₂₀ FEV ₁	0.796	0.270	0.107
Eosinophils	-0.777	0.039	-0.229
Neutrophils	-0.018	-0.241	-0.917
Macrophages	0.395	0.131	0.873
Lymphocytes	0.545	0.254	0.037
Exhaled NO	-0.804	0.064	-0.093
FEV ₁ % pred.	0.122	0.914	0.120
FEV ₁ /FVC	0.089	0.892	0.240

Loadings ≤ 0.75 are in bold.

Table 3. Factor loadings in the group of patients showing a duration of asthma > 16 years

	Factor			
	1	2	3	4
PC ₂₀ FEV ₁	0.113	0.830	-0.055	0.172
Eosinophils	-0.871	-0.118	0.109	-0.108
Neutrophils	0.220	0.000	-0.948	0.096
Macrophages	0.045	0.117	0.969	0.019
Lymphocytes	0.067	0.054	-0.048	0.980
Exhaled NO	-0.898	0.061	0.036	0.020
FEV ₁ % pred.	-0.037	0.892	0.064	0.115
FEV ₁ /FVC	-0.010	0.869	0.147	-0.246

Loadings ≤ 0.75 are in bold.

Discussion

The present study included patients with mild to moderate, extrinsic asthma without corticosteroids. In these patients airway responsiveness to methacholine was associated with airway inflammation in terms of sputum eosinophils and exhaled NO, when the duration of the disease was ≤ 16 years. In contrast, airway responsiveness was linked to baseline lung function, when patients had a longer duration of their disease.

The dissociation between airway hyper-responsiveness and inflammation and its association with lung function are suggestive of alterations that might reflect ongoing airway remodelling and structural alterations in chronic asthma.

All patients included were in a stable clinical state and had been specifically recruited by large-scale advertisement for study subjects on the basis that they did not have anti-inflammatory treatment with corticosteroids, irrespective of their clinical state. None of the patients recruited was excluded

owing to lung function or age. In those who had had inhaled corticosteroid treatment (ICS; Table 1) it was ensured that it had been ceased at least 8 weeks prior to the tests. Within each of the two groups, the subgroups previously treated with corticosteroids did not differ statistically from those never treated with corticosteroids, in terms of lung function, sputum composition, NO levels, or PC₂₀FEV₁. Moreover, the association of PC₂₀FEV₁ with either eosinophilic inflammation or lung function did not change, when patients without previous steroid treatment were excluded from the analysis. Furthermore, it is unlikely that the differences between groups were due to acute effects of the therapy with β_2 -agonists, since this was similar and none of the patients had started or stopped this therapy shortly before tests. Therefore it seems justified to assume that the previous treatment did not bias our results.

As a second issue the definition of the duration of the disease must be raised. This was asked from the subjects and defined as the interval between the time of the study and the time when the diagnosis of asthma had been established by a physician. It might be argued that the standards of asthma diagnosis have considerably changed over the years. Therefore we also asked for the time when symptoms typical for asthma had been first recognized. When the study population was divided in two groups according to the median time of onset of asthma symptoms, the pattern of factor loadings did not change. Furthermore, none of the subjects reported a complete absence of asthma symptoms over a significant period of time (> 1 year) since diagnosis. We therefore consider it unlikely that the definition of the duration of the disease or changes in the criteria of the diagnosis of asthma did bias our results in the patients studied. Naturally, the duration of the disease was linked to the subjects' age but there was no evidence that within the range studied (mean age, 26 vs. 38 years), age *per se* affected the correlation between parameters, independent of the duration of the disease.

The cut-off value of 16 years as used to define groups was derived from the requirement to have the same sample size in both groups. When a subgroup with a duration of the disease ≤ 8 years ($n = 18$) was compared with a subgroup with duration ≤ 24 years ($n = 20$), there was no qualitative change in the pattern of factor loadings. Owing to the reduction in sample size, it was not possible to perform the analysis in subgroups with an even larger separation. Patterns were also essentially maintained when the cut-off value for the group with shorter duration was increased to up to 24 years and the cut-off for the group with the longer duration was shifted down to 8 years. A median period of 16 years might seem high in view of data on early morphological changes in asthma [23] but the process of chronification of asthma, which leads to irreversible structural and functional alterations, probably comprises different levels of re-organization on different time-scales [6], ranging from the mere presence of inflammatory cells to an increase in basement membrane thickness and smooth muscle mass, epithelial shedding, and complete re-organization of the subepithelial layers. In any case, our data suggest *a posteriori* that the value of 16 years as chosen was within the sensible range and that, apparently, the change in correlation occurred on average at 10–20 years of duration of the disease.

We followed a procedure of sputum induction that had been validated previously [20]. Similarly, NO [19] and lung function [17] measurements were performed according to International Guidelines. The correlation coefficient between FEV₁% pred.

and FEV₁/FVC may be considered as a sort of best achievable value, when different but closely related aspects of the bronchial system are quantified. As compared to that upper bound, correlation coefficients between markers of airway inflammation and PC₂₀FEV₁ appeared to be rather high. They were not only significantly different from zero but also comparable to corresponding values reported in the literature. We therefore consider the lack of correlation between PC₂₀FEV₁ and eosinophils or NO levels in patients with a longer duration of the disease as true and not as a lack of data validity. Although correlation coefficients were subject to the scatter of the data and did not exhibit an all-or-nothing pattern, the values observed were different to a degree that they resulted in distinctly different patterns of factor loadings. In that respect, factor analysis was used as a means to concentrate the information contained in the data into discernible items.

Factor analysis has been applied previously in patients with asthma, but without stratification according to the duration of the disease [9]. In this study, airway responsiveness was found to be independent from airway inflammation in terms of sputum eosinophils, similarly as in the group with a longer duration of the disease. On the other hand, it was also independent from lung function, as in the group with a shorter duration of the disease. When we pooled both of our groups, the clear-cut factor loading patterns disappeared and lung function vs. eosinophils/NO were independent factors, while hyper-responsiveness was associated with both of them to about the same intermediate degree. Rosi et al. [9] also included age which they found to be weakly associated with the ECP/eosinophil factor. In our data neither age nor the duration of the disease correlated with any of the measured variables, also indicated by the fact that the mean values of these variables were similar in both groups (Table 1). Instead, the duration of the disease was associated with an alteration in the pattern of correlations between the measured variables.

The finding that neutrophils and macrophages made their own factor in both groups, is likely to be due to the fact that these cells comprised the majority of sputum cells and cell numbers had been normalized to 100%. It is known that neutrophil numbers in sputum can be elevated in patients with moderate to severe asthma [7], but the patients included in the present study showed, on average, only a mild disease (Table 1). Lymphocytes represented a separate factor in patients with a longer duration of the disease and were weakly associated with eosinophils in patients with a shorter duration; mean percentages were comparable between groups. These findings are in accordance with the fact that until now no relationship between sputum lymphocytes and functional parameters has been reported.

Airway hyper-responsiveness is thought to depend on a number of factors including smooth muscle hyper-reactivity and airway inflammation [6,24]. In asthma, inflammation is characterized by increased numbers of eosinophils, as detectable in induced sputum [2–4,7–9]. Furthermore, the levels of exhaled NO are found to be elevated [4, 8]. Although the role of NO in asthma has not been fully clarified [25,26], it seems to be a highly sensitive marker for the efficacy of anti-inflammatory treatment [1,4]. Accordingly, a relationship between sputum eosinophils and exhaled NO has been reported [8]. Since we found a significant correlation between eosinophils and NO in both groups, the relationship between these two markers of

airway inflammation did not depend on the duration of the asthmatic disease. In contrast, their relation to airway hyper-responsiveness was different, and this difference probably reflected chronic effects of asthma. The fact that this difference was observed suggests that the response to a direct stimulus such as methacholine comprises essential information on long-term changes in asthma.

The relationship between baseline lung function and airway hyper-responsiveness is well known [10–12], although not confirmed by all authors [27,28]. Interestingly, it seems to occur also in patients with chronic obstructive pulmonary disease (COPD) [29], suggesting a role for persistent structural alterations. These could act directly through a reduction in baseline airway calibre, leading to stronger lung function responses [30], whereas the reduction of calibre could reflect persistent morphological changes. Our data do not give clues on these mechanisms, however, they suggest that a long duration of the asthmatic disease was sufficient to make them more relevant. Noteworthy enough, lung function was similar in both groups but the pattern of correlation with lung function was different, thereby suggesting that the magnitude of airway obstruction was not the primary cause of the differences between groups.

In conclusion, our study demonstrates that in patients with mild to moderate asthma the relationship between airway hyper-responsiveness, markers of airway inflammation and lung function depends on the duration of the disease. These findings are suggestive of ongoing processes of airway remodeling, that lead to a dissociation between hyper-responsiveness and markers of inflammation and to its association with baseline lung function. Further studies will have to clarify whether the characteristics of these alterations can be assessed more directly, e.g. through specific markers in induced sputum or exhaled air.

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