

Original article

Assessment of inhaled BDP-dose dependency of exhaled nitric oxide and local and serum eosinophilic markers in steroids-naïve nonatopic asthmatics

The aim of the present study was to assess the dose-dependency from inhaled steroids of changes of airways inflammation [eosinophils count and eosinophil cationic protein (ECP)] measures in induced sputum and in serum, as well as that of exhaled nitric oxide. Twenty steroid-naïve patients with nonatopic asthma of mild to moderate degree [forced expiratory volume in 1 s (FEV₁) = 70% of predicted] and with negative response to the standard tests for allergy were selected; after a 1-week run-in period they were randomized to receive a 12-week treatment period of inhaled beclomethasone dipropionate dry powder given with the Pulvinal® inhaler (Clenil P®, Chiesi Farmaceutici S.p.A., Parma, Italy) in two different dose regimens, 400 µg bid (high dose) or 200 µg bid (low dose), over a double blind, parallel groups design. The following outcome measures were assessed in baseline and after 1, 6 and 12 weeks of treatment: FEV₁ (l), eosinophils count in sputum (%), is ECP (µg/l), serum eosinophils count (%), serum ECP (µg/l) and exhaled NO (ppb). The results showed that all the considered parameters improved in both groups: the increase over baseline of FEV₁ and the decrease of NO were significant at any time in the high-dose group and only at week 12 in the low-dose group (NS between groups), whereas the markers of eosinophilic activity showed more consistent reductions in the high-dose than in the low-dose group when measured in induced sputum ($P < 0.05$ between groups after 6 and 12 weeks for eosinophils count and after 12 weeks for ECP). Decreases over baseline of markers measured in serum were more rapid in the high-dose group, without differences between groups. A marked trend towards a negative correlation was found between FEV₁ and ECP, ($r = -0.72$, $P < 0.05$), between FEV₁ and eosinophils in sputum ($r = -0.31$, NS) and between FEV₁ and exhaled NO ($r = -0.38$, NS), all of them only in the high-dose group. The results of the study demonstrate that changes of levels of eosinophilic activity in the airways are dependent from the daily dose of inhaled steroids when measured in induced sputum and that the local assessment can therefore represent a practical and noninvasive method to monitor the extent of airways inflammation.

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The effects of eosinophils in the pathogenesis of airways inflammation and their role in asthma have been well defined (1). It has been demonstrated that their number and the level of eosinophil chemotactic factors (2) are increased in atopic asthma during the pollen season and that they correlate with the severity of asthma as measured by standard pulmonary function tests (1). In addition, it has also been recently reported that the serum levels of the eosinophil cationic protein (ECP), which is considered a reliable marker of eosinophilic activity, are good indicators of a dose–response effect after treatment with inhaled corticosteroids (3). In terms of a possible role of the measurement of ECP in the clinical practice, it is well known that it is a sensitive marker of airflow

obstruction in chronic asthma and that the elevated levels of ECP, especially when locally assessed (i.e. in induced sputum), identify patients at risk of inflammatory exacerbations (4). These findings may have relevant practical implications in the management of chronic asthma, as the elevated ECP levels most likely represent asthmatic patients nonadequately treated. Among the methods of assessment of ECP, it has been showed that the local measurement of ECP in sputum is a more accurate marker of asthmatic airway inflammation than the proportion of blood eosinophils count or serum ECP (5), being the sputum ECP levels more closely related to lung function parameters than those measured in peripheral blood (6).

More recently, an emerging role of exhaled nitric oxide (eNO) has been also proposed as noninvasive marker of airways inflammation in asthma. Recent findings suggest that the exhaled NO levels and the eosinophils count in sputum are significantly correlated in steroid-naïve asthmatic, and that this relationship is less evident in steroid-treated patients (7) and in healthy subjects (8). In addition it has been demonstrated that the exhaled NO levels are mainly correlated with markers of asthma inflammation, including bronchial hyperreactivity, and markers of asthma control (i.e. peak expiratory flow rate (PEFR) diurnal variability), whereas the correlation with the severity of the disease, as expressed by FEV₁ % predicted, is less evident (9). However, the raised levels of exhaled NO have been mainly found in atopic subjects with asthma (10) or in allergic children (11), rather than in nonatopic asthmatic adults.

On the basis of these recent findings, we wanted to investigate the effects of two dose levels of inhaled beclomethasone dipropionate (BDP) on exhaled NO and on local and serum levels of eosinophilic markers in nonatopic steroid-naïve asthmatic adults. A secondary objective was the assessment of the correlation between the effects of the treatment on pulmonary function test and on markers of inflammation.

Patients and methods

Study population

Twenty adult patients with nonatopic asthma were recruited from the Lung Department of the Hospital of Bussolengo, Verona (Italy). Patients were admitted to enter the run-in period if they met the following criteria: males or females aged between 18 and 60 years; diagnosis of mild to moderate persistent asthma defined according with the criteria of the National Heart Lung and Blood Institute (12); forced expiratory volume in 1 s (FEV₁) of at least 70% of predicted normal; positive response to the reversibility test defined as an increase of at least 15% after administration of 200 µg inhaled salbutamol; negative response to Prick (set of the 24 most common inhalant allergens), Prist or radioallergosorbent test (done at study entry, for 12 most common foods) for any known allergen. Exclusion criteria were as follows: use of steroids or cromoglycate (by any route) in the 8 weeks preceding the qualification visit; use of theophylline and/or long acting β₂-agonists in the last 4 weeks; patients with asthma exacerbation and/or upper or lower respiratory tract infections in the last 4 weeks; patients with other serious underlying diseases; patients with other pulmonary diseases to asthma (i.e. bronchiectasis, fibrosis, pulmonary hypertension, emphysema, chronic bronchitis, α₁-antitrypsin deficiency) which could interfere with the study results; pregnancy or females at risk of pregnancy (i.e. those not demonstrating adequate contraception). Patients who had an asthma exacerbation during the run-in and required a treatment different to that given in the present study were also excluded from taking part in the treatment phase.

Study design and protocol

Eligible patients entered a 2-week run-in period and were then randomized to receive a 12-week treatment with BDP dry powder given via the Pulvinal® inhaler (Clenil P®, Chiesi Farmaceutici

S.p.A., Parma, Italy) in two different doses, 400 µg bid (high dose) or 200 µg bid (low dose), following a double blind, parallel groups design. Inhaled salbutamol was permitted as rescue medication during the study period.

Pulvinal® is a novel dry powder inhaler (DPI) designed to deliver inhaled drugs to the airways, which is classified as a high-resistance device. Its resistance to flow is higher than that of other DPIs such as the Turbohaler® (intermediate) or the Rotahaler® (low-resistance device). Previous *in vitro* and *in vivo* data suggested that the peak inspiratory flow rate (PIFR) measured through the Pulvinal® was independent of the severity of the expiratory airflow obstruction, and that also the lowest PIFR values proved sufficient to deliver an effective bronchodilator dose (13).

Visits at the clinic took place between 08.00 and 10.00 AM at study entry, at the end of run-in/start of treatment and after 1, 6 and 12 weeks of treatment.

The FEV₁ (% of predicted normal) was measured at each visit using a whole body plethysmograph (Masterlab Body®, Jaeger, Germany); measuring conditions, equipment and daily calibration of the instrument were performed according to standard guidelines (14). Three consecutive tests were carried out and the best was recorded. If salbutamol had been administered, a minimum of 6 h had to elapse between the intake and the pulmonary function test.

In addition, the following lab-tests were measured at each visit: blood eosinophils count (bEOS, %), induced sputum eosinophils count (isEOS, %), serum eosinophil cationic protein (sECP, µg/l), induced sputum ECP (isECP, µg/l) and eNO (ppb).

A 10 ml blood sample was taken between 08.00–09.00 AM from an antecubital vein via the Becton & Dickinson Vacutainer® System Europe (Meylan, Cedex, France) to assess the bEOS and the sECP levels. The eosinophils count was performed using an automated haematological analyser (SE 9000®, Bayer, Milan, Italy). The assessment of ECP was done by immune-fluorescence assay (CAP System ECP FEIA®, Kabi Pharmacia Diagnostic, Uppsala, Sweden). Blood samples drawn from an antecubital vein were thermostated at 22°C for the clotting time (60 min) and then exposed to double centrifugation in order to avoid the presence of residual eosinophils in serum. For the assessment of sECP, samples were kept at 22°C for the clotting time and were then exposed to a double centrifugation to remove residual eosinophils from serum (15).

The sputum induction was performed with an inhalation of hypertonic saline solution for up to 30 min after premedication with 200 µg inhaled salbutamol. Subjects were asked to expectorate every 5 min: the portions of fresh sputum were selected to exclude salivary contamination and the cell count was then performed (16).

The assessment of exhaled NO was performed after a single and prolonged expiration at a constant flow of 6 l/min of 30 s duration; subjects were invited to exhale against a resistance to determine the closure of the soft palate and thereby to avoid the contamination with the NO of nasal cavities. The level of eNO was assessed via a chemoluminescence analyser (NO Analyser, SensorMedics Italia, Milan, Italy).

Ethics

Patients gave their written informed consent before any study-related procedure was started. The study protocol was approved by the local Ethics Committee.

Statistics

Values measured at the end of run-in/start of treatment were considered as baseline. The homogeneity of baseline values was

analysed using the unpaired Student's *t*-test; the comparison within or between groups was performed using paired and unpaired *t*-test, respectively, for all the considered variables. The Pearson's coefficient was used to assess the correlation between FEV₁ and laboratory parameters. The level of significance was set at *P* < 0.05.

Results

The study population was made of 20 patients in total (mean age 45.5 years), 10 in either group. All randomized patients completed the 12-week study period.

Data collected at study entry are showed in Table 1. The two groups were well matched for demographics, baseline values of FEV₁ and laboratory parameters. The results at

each time point for all variables are showed in Table 2. The individual patients' data of outcomes measured in the induced sputum collected at baseline and after 6 and 12 weeks are showed in Fig. 1 (isEOS) and Fig. 2 (isECP).

The FEV₁ improved in both groups: the increases were more evident in the high-dose group, where the level of statistical significance over baseline was reached just after 1 week of treatment (*P* < 0.05) and was maintained after 6 and 12 weeks (*P* < 0.01 in both cases); in the low dose group the level of significance was reached at the end of treatment (*P* < 0.05). The comparison between groups did not show statistically significant differences at any time point.

The sEOS showed significant increases over baseline in both groups (low-dose: *P* < 0.05 after 6 and 12 weeks; high dose: *P* < 0.01 after 1 and 6 weeks, *P* < 0.05 after 12 weeks), without differences in the comparison between groups.

The serum ECP levels similarly decreased in both groups: the level of significance was reached only in the low-dose group at the end of treatment, without differences between groups at any time point.

The results of the variables measured in the induced sputum showed statistically significant differences between the two dose regimens for both variables. In fact, the decrease of local eosinophils count (Fig. 1) was more marked in the high-dose group (*P* < 0.05 over baseline after 1 week and *P* < 0.01 after 6 and 12 weeks) than in the low-dose group (*P* < 0.05 after 1 week, *P* < 0.01 after 6 weeks and NS at the end of treatment); the comparison between groups showed a statistically significant difference (*P* < 0.05) after 6 and 12 weeks.

Table 1. Baseline characteristics of the patients' population

	Low dose (n = 10)	High dose (n = 10)
Sex		
Males (n)	4	5
Females (n)	6	5
Age (years)	43.9 ± 16.7	47.2 ± 12.4
FEV ₁ predicted (%)	88.06 ± 13.9	84.67 ± 11.8
Serum eosinophils (%)	8.51 ± 4.1	6.42 ± 2.6
Serum ECP (μg/l)	20.32 ± 17.6	17.09 ± 21.6
Is eosinophils (%)	41.60 ± 24.6	46.40 ± 33.3
isECP (μg/l)	2270.90 ± 2500.7	2154.9 ± 3141.3
Exhaled NO (ppb)	70.61 ± 36.7	69.48 ± 34.5

Values are expressed as mean ± SD. NS between groups for all parameters. FEV₁, forced expiratory volume in 1 s; ECP, eosinophil cationic protein; isECP, induced sputum ECP; NO, nitric oxide.

Table 2. Results in the two groups at each time-point

Treatment	Baseline (mean ± SD)	After 1 week (mean ± SD)	After 6 weeks (mean ± SD)	After 12 weeks (mean ± SD)
FEV ₁ predicted (%)				
High dose	87.50 ± 9.73	95.39 ± 9.35*	90.71 ± 8.31**	92.28 ± 8.60**
Low dose	86.01 ± 12.43	87.71 ± 14.59	91.25 ± 11.99	94.77 ± 10.74*
Serum eosinophils (%)				
High dose	6.70 ± 2.89	5.19 ± 2.23**	5.16 ± 1.95**	4.76 ± 2.25*
Low dose	8.38 ± 3.79	8.12 ± 3.88	6.64 ± 3.21*	6.38 ± 2.70*
Serum ECP (μg/l)				
High dose	19.08 ± 24.45	10.41 ± 11.50	9.20 ± 10.08	8.52 ± 7.29
Low dose	20.81 ± 17.55	18.76 ± 18.75	10.75 ± 9.70	9.39 ± 9.52*
Is eosinophils (%)				
High dose	44.44 ± 32.03	27.68 ± 28.05*	27.40 ± 28.75*****	18.50 ± 21.01*****
Low dose	37.60 ± 20.02	33.40 ± 19.42*	31.20 ± 20.19**	34.40 ± 27.21
isECP (μg/l)				
High dose	2262.10 ± 3275.53	1105.24 ± 2037.68	857.70 ± 1604.07	443.26 ± 920.38***
Low dose	2391.70 ± 2825.42	1063.60 ± 843.91	1167.20 ± 1147.32	1051.70 ± 725.75
Exhaled NO (ppb)				
High dose	72.00 ± 33.63	49.82 ± 23.82*	40.91 ± 16.56**	33.50 ± 13.65**
Low dose	74.75 ± 47.96	60.94 ± 47.31	50.38 ± 45.56	26.65 ± 11.37**

* *P* < 0.05, ** *P* < 0.01 vs baseline, *** *P* < 0.05 between groups. Values are expressed as mean ± SD. FEV₁, forced expiratory volume in 1 s; ECP, eosinophil cationic protein; isECP, induced sputum ECP; NO, nitric oxide.

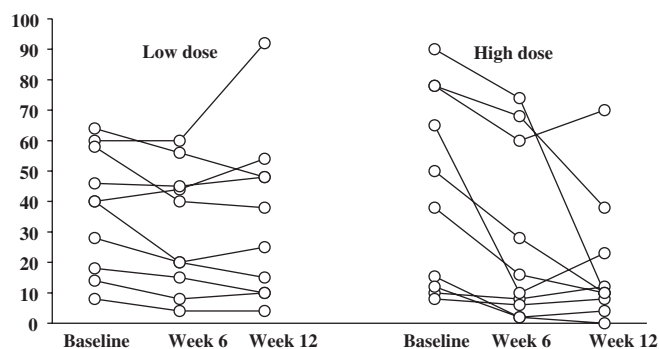


Figure 1. Induced sputum eosinophils count (% total leucocytes): individual patients' data in the two groups. $P < 0.05$ between groups at weeks 6 and 12.

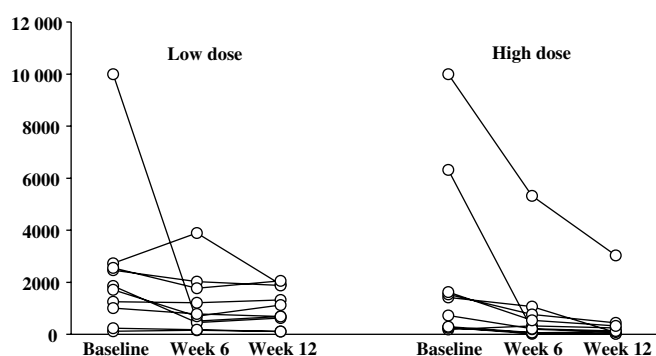


Figure 2. Induced sputum ECP ($\mu\text{g/l}$): individual patients' data in the two groups. $P < 0.05$ between groups at week 12.

Similarly, the results of isECP (Fig. 2) showed that more marked decreases over baseline occurred in the high-dose group ($P = 0.06$ after 1 and 6 weeks, $P < 0.05$ after 12 weeks) than in the low-dose group (NS at each time points); the comparison between groups showed a significant difference at the end of treatment ($P < 0.05$).

The decreases of levels of eNO were also more evident in the high-dose group ($P < 0.05$ after 1 week, $P < 0.01$ after 6 and 12 weeks) than in the low-dose group ($P < 0.01$ after 2 weeks), without differences in the comparisons between them.

The analysis of correlation coefficients showed a statistically significant negative correlation between FEV_1 and isECP in the high-dose group ($r = -0.72$, $P < 0.05$); a marked trend towards a negative correlation was also found between FEV_1 and eosinophils in sputum ($r = -0.31$) and between FEV_1 and eNO ($r = -0.38$) in the high-dose group, which did not reach the level of significance in both cases. No correlation was found between FEV_1 and the other variables in the low-dose group.

Discussion

Taking into consideration that the eosinophilic inflammation is a relevant feature of asthma and that the measure-

ment of eosinophils and ECP in sputum or in peripheral blood is a reliable marker which correlates with value of lung function parameters (17), we wanted to investigate if changes of such markers can be sensitive of a dose effect of inhaled BDP dry powder. In addition, the reliability of eosinophilic activity markers has been mainly demonstrated in atopic asthma and in children (18, 19); in the present trial a patients' population with nonatopic asthma has been selected to assess the dose-response effects in this specific sample. On the contrary, based on the evidence that the measurement of exhaled NO is useful to assess the airways inflammation and its severity in steroid-naive patients, whereas this marker seems to have a little relevance in a steroid-dependent population (7), a sample of patients who had abstained from use of corticosteroids in the 8 weeks prior to enrolment was selected.

The results of the study show that the effects of treatment with higher dose (800 $\mu\text{g/day}$) or lower dose (400 $\mu\text{g/day}$) of inhaled BDP, both doses given in a bid regimen, are dose-dependent when the markers of inflammation are locally measured (i.e. eosinophils count and ECP in induced sputum), as demonstrated by a statistically significant difference between groups ($P < 0.05$) after 12 weeks of treatment for eosinophils count and after 6 and 12 weeks for isECP. As far as the other variables are concerned (FEV_1 , serum eosinophils and ECP, eNO), a more rapid improvement was reported in the high-dose group; the comparison between groups did not point into evidence any significant difference for all these latter variables. In addition, a marked trend towards a negative correlation between $\text{FEV}_1\%$ predicted and markers in induced sputum, as well as between $\text{FEV}_1\%$ predicted and exhaled NO, has been shown only in the high-dose group ($P < 0.05$ between FEV_1 and isECP).

These findings confirm that, other than in atopic asthma, the monitoring of eosinophilic airway inflammation may also be useful in the assessment of the effects of inhaled steroids in patients without history of allergy. In addition, it is confirmed that the measurement of markers in induced sputum is a more reliable indicator of the effects of the anti-inflammatory treatment. As a consequence, the reliability of the levels of local markers may also be helpful in the management of initial treatment of asthma and for the titration of anti-inflammatory treatment in the long-term course.

Despite the fact that the sample population used in the present trial (10 patients in each group) is quite reduced, these results, according with that reported in literature (20), confirm that the levels of eNO accompany the other indices of inflammation in asthma; nevertheless, the measurements of local markers of eosinophils activity seems to be more sensitive than exhaled NO in the assessment a dose-effect of inhaled steroids. The lack of correlation between the effects on pulmonary function test and markers in the low-dose treatment group may be explained on the basis of a reduced room for improvement due to both the low severity of asthma and the low daily

dose; in addition, it has been reported that nonatopic subjects with asthma have lower baseline levels of eNO than atopic patients (10). Despite these baseline conditions, a clear evidence of dose-related outcome has been demonstrated in the rapidity of onset of effects: in fact, changes in FEV₁ and inflammation markers were significant over baseline only in the high-dose group for most of parameters.

In conclusion, the results of the present study show that the measurement of markers of airways inflammation taken from local samples is a practical and noninvasive method to assess the dose-dependent effects of inhaled corticosteroids; further studies with higher patients' samples would be useful to confirm their role in the long-term management of inflammatory airways diseases.

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