

Eosinophil markers in blood, serum, and urine for monitoring the clinical course in childhood asthma: Impact of budesonide treatment and withdrawal

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Background: Markers of airway inflammation are needed for prediction of asthma deterioration and evaluation of disease severity. Few studies have focused on the dynamics of airway inflammation as reflected by the activity of the eosinophils and their proteins after withdrawal of inhaled corticosteroids.

Objective: Our goal was to investigate the effect of withdrawal of inhaled budesonide on eosinophil count in blood and eosinophil proteins in serum and urine and to relate the levels of these markers to the risk of symptoms of asthma, increased bronchial hyperresponsiveness, and deterioration of lung function.

Methods: Thirty-three children were randomly selected to continue or discontinue use of inhaled budesonide in a double-blind, placebo-controlled study. They were followed up for 4 months with regular analysis of blood, serum, and urine samples; lung function; and methacholine challenges. Eosinophil activity markers were analyzed. Age-matched healthy children provided reference data for all parameters measured.

Results: The eosinophil number in blood and eosinophil protein levels in serum (serum eosinophil cationic protein [ECP] and serum eosinophil peroxidase [EPO]) increased significantly in the withdrawal group, and the difference between the groups was significant ($P = .02$ for all). Twenty-nine percent of the children in the withdrawal group remained symptom free. This subgroup had eosinophil counts at baseline below $350/\mu\text{L}$, a serum ECP level below $15 \mu\text{g/L}$, and a serum EPO level below $25 \mu\text{g/L}$, each of which was related to a low risk of exacerbation (relative risk = 0.37, 0.48, and 0.37 respectively; $P < .05$ for all). All eosinophil markers were lower in the healthy children than in the symptom-free children with asthma.

Conclusion: Our data indicate that eosinophil count and/or ECP and EPO levels can be used to estimate the short-term risk of deterioration and the need for corticosteroid treatment in cases of mild and moderate allergic asthma. (*J Allergy Clin Immunol* 2001;107:812-7.)

Key words: Budesonide, withdrawal, cessation, eosinophil cationic protein, eosinophil peroxidase, eosinophil protein X, eosinophils, children, asthma, animal dander allergy, bronchial hyperresponsiveness

Asthma is a chronic inflammatory disease in which inflammatory cells, mainly eosinophils, accumulate in the airway mucosa.^{1,2} Previous observations have shown that a high peripheral blood eosinophil (PBE) count indicates an increased risk of relapse in acute asthma³ and that the activation state of PBEs may correlate with lung function⁴ and disease severity.^{5,6}

Eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil protein X/eosinophil-derived neurotoxin (EPX/EDN) have been used as markers of ongoing eosinophil inflammation⁷ and for prediction of the clinical course of asthma.^{7,8} In addition, both the expression of EG2 epitope on intracellular ECP and the surface expression of CD9 have been suggested to be positively correlated with asthma.^{9,10}

Regular treatment with inhaled corticosteroids leads to a decrease in the number of inflammatory cells and improvement of asthma symptoms and lung function,¹¹ although in some studies this relationship is not so obvious.¹² Treatment with inhaled steroids may give long-lasting amelioration of symptoms, improved lung function, and reduced bronchial hyperresponsiveness (BHR) even after cessation of therapy.^{13,14} Fewer data are available on the impact of withdrawal on the clinical course and the airway inflammation as reflected by inflammatory markers. The aim of this placebo-controlled study was to relate asthma deterioration and clinical symptoms to lung function, inflammatory markers, and BHR, in children with allergic asthma, after withdrawal of inhaled corticosteroids. We also sought to characterize the individual response and course of disease in those who relapsed and those who remained symptom free.

METHODS

Study design

The study lasted 5 months and consisted of a run-in period (2-6 weeks) and an intervention period (4 months). Symptom control for at least 3 months and regular treatment with inhaled budesonide were required and then confirmed during the run-in period. The children were randomly grouped in a double-blind and parallel manner either to continue on budesonide (the continuous treatment group) or to receive placebo (the withdrawal group). The children

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Abbreviations used

BHR:	Bronchial hyperresponsiveness
ECP:	Eosinophil cationic protein
EPO:	Eosinophil peroxidase
EPX/EDN:	Eosinophil protein X/eosinophil-derived neurotoxin
MPO:	Myeloperoxidase
OR:	Odds ratio
PBE:	Peripheral blood eosinophil
PD15:	Provocative dose that causes a 15% fall in FEV ₁
RR:	Relative risk
SPT:	Skin prick test

came for 6 visits during 15 weeks. They were allowed to take 1 oral dose (4 mg) of betamethasone as rescue medicine if worsening of asthma occurred. They were withdrawn from the study if they experienced daily symptoms during normal activity, a need for β_2 -agonist more than 4 times a day, an irreversible 20% decrease in FEV₁, and/or more than a 20% decrease in PEF for more than 3 days. The study period was outside the pollen season, from October to the beginning of April. The children were assessed at each visit, between 7 and 9 AM. Physical examination and lung function tests were performed and blood was drawn at every visit. The children underwent methacholine challenge at run-in and 5, 9, and 15 weeks after randomization.

Subjects

The inclusion criteria were aged between 9 and 16 years, history of mild-to-moderate asthma and allergy to cat and/or dog confirmed by a positive skin prick test (SPT) result, daily use of budesonide 200 or 400 μ g for more than 3 months, and FEV₁ greater than 80% of predicted. The children were excluded if they were sensitized to house dust mite or to a family pet. One child had a dog; the others had no furry pets.

A total of 34 children were included. Randomization was stratified for different doses of budesonide (400 μ g/d or 200 μ g/d). One girl had a severe infection on the day of the randomization and therefore never entered the study. The remaining 33 children (Table I) participated according to the study protocol.

Healthy control subjects

Sixteen age-matched, healthy children served as control subjects (Table I). To exclude atopy, SPT for cat, dog, horse, house dust mite, molds, birch, grass, and mugwort was done. Inflammatory markers in blood and urine, lung function, and bronchial responsiveness to methacholine were examined once.

Symptom score

Symptoms were recorded daily (wheezing, cough, dyspnea, and exercise-induced asthma). At each visit the symptom diary was evaluated by the patient together with the physician, and symptoms were stratified on a scale of 1 to 3 (1 = no symptoms; 2 = symptoms, no rescue medication required; 3 = symptoms, either rescue medication or exclusion from the study according to previously described criteria).

Skin prick test

SPT was done with extracts of cat, dog, horse, rat, and rabbit (Soluprick 10.000 SQ; ALK, Hørsholm, Denmark) in duplicate. A mean wheal diameter of more than 3 mm was considered positive.

TABLE I. Characteristics of patients and control subjects at baseline

	Continuous treatment group	Withdrawal group	Healthy controls
n	16	17	16
Mean age (y)	12.3	11.8	13.3
Sex (F/M)	5/11	11/6	10/6
Budesonide 400/200 μ g	13/3	13/4	0
Positive SPT for cat	16	17	0
Positive SPT for dog	15	15	0

Spirometry and methacholine challenge

Spirometry was performed by means of flow-volume curves (Vitalograph). Methacholine challenge was performed according to the protocol of Chai et al¹⁵ with slight modifications. The children inhaled increasing concentrations of methacholine delivered by a dosimeter (Spira Elektro 2 nebulizer system; Respiratory Care Center, Hamenlinna, Finland). Five breaths at each concentration resulted in cumulative doses of methacholine from a minimum of 22 μ g to a maximum of 2830 μ g. The challenge was stopped when a greater than 15% fall in FEV₁ occurred or the maximum dose was reached. The bronchial reactivity to methacholine (PD15 [provocative dose that causes a 15% fall in FEV₁]) was determined from the log dose-response curve by linear interpolation.

Markers of inflammation

At all visits the following samples were collected: 5 mL EDTA-blood for flow cytometry analysis, and serum for ECP, EPO, EPX/EDN, and myeloperoxidase (MPO) measurements. Sera were handled according to the suppliers of the ECP kit. Urine specimens were collected from the first morning portion at home and kept in the refrigerator until being transferred to the hospital. Sera and urine samples were stored at -20°C until analysis.

Serum ECP, serum EPO, serum MPO, serum EPX/EDN, and urinary EPX/EDN

Serum (S)-ECP was measured with a commercially available fluoroimmunoassay (Pharmacia ECP CAP System FEIA; Pharmacia & Upjohn, Uppsala, Sweden). The detection limit was 2 μ g/L. S-EPO was assessed with a prototype immunofluorometric assay, with the Pharmacia CAP system. The EPO standard curve of Carlson et al¹⁶ was used and covered the range from 0.5 to 200 μ g/L. S-MPO was measured by RIA (Pharmacia & Upjohn).¹⁷ The detection limit was 8 μ g/L. S-EPX and urinary (U)-EPX were determined by a specific, competitive RIA (Pharmacia & Upjohn). U-EPX was adjusted to creatinine levels in urine. All interassay coefficients of variation were below 13%.

Number of PBEs and expression of CD9 and EG2 epitope

For flow cytometric analysis of PBEs in unseparated blood, a cell membrane permeabilization technique (the FOG method) was used as previously described in detail.^{18,19} The expression of CD9 and the intracellular EG2 epitope on ECP were measured in specific mean fluorescence intensity units.

Statistical methods

To evaluate the individual changes during the study period, baseline values for each subject were subtracted from the corresponding value at the following visits. Comparison between the groups at the separate visits was done with the Mann-Whitney *U* test. Multiple linear regression was used to assess changes in parameters through the

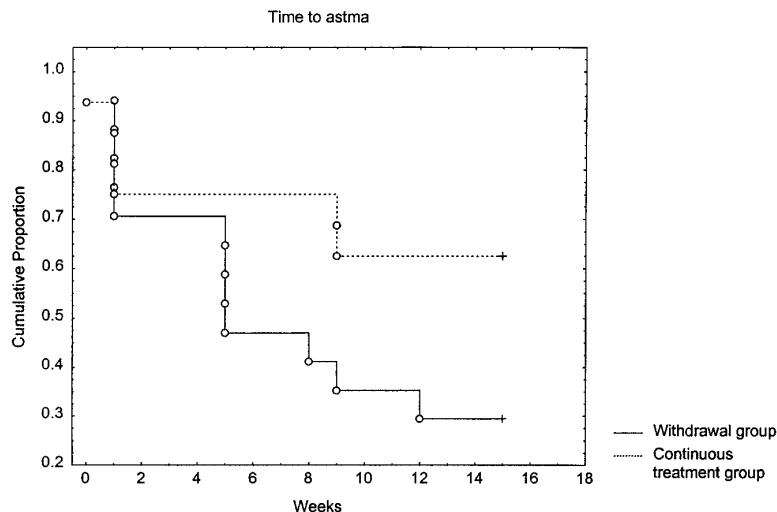


FIG 1. Cumulative proportion of children with exacerbation of symptoms and the difference between the withdrawal and the continuous treatment groups.

study period, and the unpaired Student *t* test was used to compare differences between the groups. Changes from baseline within the separate groups were calculated by the Wilcoxon paired rank test. Results are presented as medians (interquartile ranges). Relationships between variables were evaluated by the Spearman rank correlation coefficient. Time to deterioration was described by the Kaplan-Meier survival curve, and the risk of deterioration was analyzed with the χ^2 test. The logistic regression model was used to evaluate odds ratios (OR and 95% CI) for parameters as continuous variables. Crude risk ratios (relative risk [RR] and 95% CI) for development of symptoms in the subgroups were calculated for defined cutoff points of different markers, and differences were calculated with the Fisher exact test; $P < .05$ was considered significant.

Informed consent

The study was approved by the local ethics committee. Informed consent was obtained from both children and parents.

RESULTS

Subjects

Thirty-three children were included in the study. Sixteen children were randomly assigned to the continuous treatment group, and 17 children were assigned to the withdrawal group (Table I). Three children assigned to the withdrawal group were later excluded from the study because of exacerbation of asthma symptoms despite 1 dose of betamethasone, compared with none in the continuous treatment group. The remaining 30 children attended all visits and participated in all required measurements.

Symptom score

All children were symptom free at entry. In the withdrawal group, 71% (12/17) of the children got symptoms of asthma and 47% (8/17) received 1 dose of rescue medication (betamethasone 4 mg), whereas in the continuous treatment group, the corresponding figures were 38% (6/16) and 38% (6/16), respectively. The difference between the groups was significant ($P < .05$) for symptoms.

The time to exacerbation is presented in the Kaplan-Meier survival curve (Fig 1). Twenty-nine percent of the children in the withdrawal group and 62% in the continuous treatment group remained symptom free throughout the study.

Lung function and BHR

The change in lung function (FEV_1) was significantly different between the groups at all visits ($P < .02$) (data not shown) but did not correlate with any inflammatory parameter. Children in the withdrawal group had decreased FEV_1 already after 1 week and then no further change was seen within this group, whereas there was an increase in FEV_1 in the continuous treatment group after 5 weeks, which remained until the end of the study.

The children with asthma had a median PD15 of 280 μ g, but the change in PD15 was not significant, and no difference was observed between the continuous treatment group and the withdrawal group. The 3 children in the withdrawal group who dropped out because of exacerbation of asthma performed only baseline tests.

Markers of inflammation

PBE count. The number of PBEs and the percentage of eosinophils (PBE/total cell count) at baseline are presented in Table II. No significant difference was found at baseline between the children randomly assigned to the continuous treatment group and those randomly assigned to the withdrawal group. Children in the withdrawal group had a significantly increased PBE count already after 1 week of withdrawal ($P < .05$), and this change within the group was also significant after 15 weeks ($P < .05$). The same tendency was observed 9 weeks after withdrawal ($P = .069$) (Fig 2). The children in the continuous treatment group had a transient decrease in PBE count after 5 and 9 weeks ($P < .05$) but not at the other visits. When all the data were pooled, the change measured by linear regression analysis was significantly

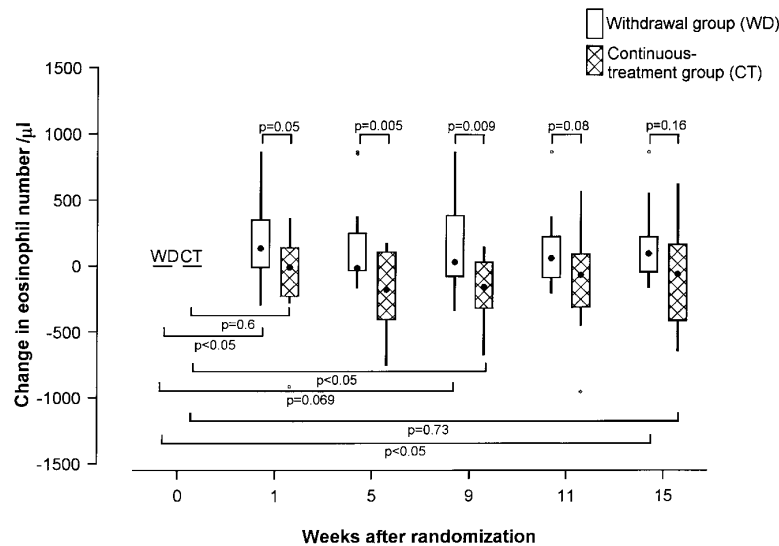


FIG 2. Change in PBE number compared with baseline. The change from baseline values and differences between the groups are presented as median, interquartile range, minimum/maximum, and outliers (dots). The changes within the separate groups are presented with *square brackets* below the boxes and are based on absolute values.

TABLE II. Inflammatory markers at baseline, in relation to symptom development in patient groups

	No symptoms		Symptoms		
	Withdrawal group (n = 5)	Continuous treatment group (n = 9)	Withdrawal group (n = 12)	Continuous treatment group (n = 6)	Healthy controls (n = 16)
Eosinophil count (μL^{-1})	235 (180-372)	645 (120-728)	528 (203-1778)	478 (294-2012)	236 (74-345)
Eosinophils (%)	4.6 (3.6-8.0)	10.2 (3.2-16.8)	7.2 (5.0-22.8)	9.9 (4.6-27.5)	4.4 (1.1-8.8)
S-ECP ($\mu\text{g/L}$)	14 (7-26)	20 (8-37)	23 (12-144)	22 (11-89)	12 (3-24)
S-EPO ($\mu\text{g/L}$)	18 (13-45)	33 (10-71)	35 (21-221)	34 (14-83)	15 (2-36)
U-EPX ($\mu\text{g/mmol creatinine}$)	104 (50-122)	161 (67-282)	191 (50-426)	135 (68-435)	68 (31-204)

Data are presented as median (range).

more pronounced in the withdrawal than in the continuous treatment group ($P < .01$).

The 5 children who remained symptom free in the withdrawal group had a significantly lower ($P < .05$) number of PBEs at baseline (median, 235 PBE/ μL) than the 12 children who had symptoms after withdrawal of budesonide (median, 528 PBE/ μL) (Table II). When baseline PBE count and the percentage of eosinophils were related to the risk of exacerbation in the withdrawal group, a PBE count less than 350/ μL or less than 6% eosinophils indicated a significantly lower risk of symptom development (RR 0.37 [0.12-1.15] and $P = .028$ for both). However, if budesonide treatment was continued, this relation was not prognostic. The risk ratio for exacerbation if the PBE count was greater than 700/ μL was 1.56 (1.1-2.30); $P = .22$ for the withdrawal group. Two of the 3 dropouts had PBE counts greater than 1000/ μL at baseline.

Serum ECP, EPO, EPX/EDN, and urinary EPX/EDN. At randomization, there was no significant difference between the groups in S-ECP, S-EPO, S-EPX, or U-EPX. At the first visit after withdrawal of budesonide, levels of eosinophil proteins in serum increased and became sig-

nificantly higher than those in the continuous treatment group. The S-ECP and the S-EPO levels in the withdrawal group differed significantly from those in the continuous treatment group at 1 and 5 weeks ($P = .04$ and $P = .057$, respectively, for ECP and $P = .017$ and $P = .03$, respectively, for EPO) but did not change further. When all data were pooled, the overall change measured by linear regression analysis was significant for S-ECP ($P < .01$) and S-EPO ($P = .02$), but not for S-EPX and U-EPX, in the withdrawal group compared with the continuous treatment group.

Low levels of all eosinophil markers at baseline were associated with low risk of exacerbation in the withdrawal group. S-ECP below 16 $\mu\text{g/L}$, S-EPO below 26 $\mu\text{g/L}$, S-EPX/EDN below 56 $\mu\text{g/L}$, and U-EPX/EDN below 126 $\mu\text{g/mmol creatinine}$ at baseline were all related to lower risk of later symptoms (RR = 0.48, 0.37, 0.50, and 0.44, respectively; $P < .03$ for all markers). The OR for developing asthma symptoms increased with increasing baseline levels of PBE, S-ECP, or U-EPX in both groups; however, the number of children was too small for the difference to reach significance levels of $P < .05$ (Table III).

TABLE III. Logistic regression at baseline

Parameter	Intervals	OR*	Lower	Upper
Eosinophil count (μL^{-1})	100	1.20	0.94	1.55
Eosinophils (%)	2	1.28	0.89	1.85
S-ECP ($\mu\text{g/L}$)	10	1.76	0.87	3.59
U-EPX ($\mu\text{g}/\text{mmol creatinine}$)	20	1.13	0.92	1.38

Results are presented as OR with the 95% CI.

*Adjusted for sex, age, and treatment.

Peripheral total blood cell count and S-MPO. The total blood cell count and the levels of S-MPO did not change after withdrawal of budesonide.

Expression of CD9 and EG2. The change in the expression of EG2 and CD9 was not significant at the separate visits. There were no significant changes within the separate groups. When all data were pooled, there was a significant difference between the 2 groups: there was an increase in the expression of the EG2 epitope on intracellular ECP in PBEs ($P = .012$), measured by linear regression, and a trend ($P = .059$) toward increased CD9 surface expression in the withdrawal group compared with the continuous treatment group (data not shown).

Healthy control subjects. Baseline values for PBE, S-ECP, S-EPO, S-EPX/EDN, and U-EPX/EDN were all significantly lower in the healthy control children than in the asthmatic children ($P < .01$) (Table II). Lung function, measured by FEV_{10} , was within the normal range in healthy children, and their median methacholine PD15 was greater than 2800 μg ($P < .001$ compared with the children with asthma). Two healthy children had PD15 values below 700 μg .

DISCUSSION

To our knowledge, this is the first double-blind, randomized, placebo-controlled study that addresses the impact of withdrawal of inhaled corticosteroids on airway inflammation in children with allergic asthma. The results demonstrate that withdrawal of a low dose of inhaled budesonide induces deterioration and increased airway inflammation measured by inflammatory markers in blood and serum. The children were all allergic to animal dander and thus were naturally exposed to allergen in their daily surroundings.²⁰ This exposure implies a risk for allergic airway inflammation, especially in those not treated with inhaled steroids. One week after randomization to continued budesonide treatment or withdrawal, there was already a significant increase in eosinophil count in the withdrawal group and an increase in S-ECP and S-EPO levels. The magnitude of this change varied during the study. Possible explanations for this inconsistency are the changeable nature of the disease, such as acute exacerbations and the single allowed oral dose of corticosteroids, which could have blunted the inflammatory response.^{21,22}

The compliance to treatment—whether corticosteroid or placebo—was probably increased in both groups, as in most studies. This might have resulted in an improve-

ment in the children in the continuous treatment group. This effect may well have been reflected in the transient decrease in PBE count and serum markers in the continuous treatment group, which augmented the difference between the groups during part of the study time. Still, there was a difference in levels of eosinophil activity markers throughout the study, and at the end of the study, inflammatory serum markers were also significantly increased within the withdrawal group.

We found low baseline values for eosinophil count as well as for S-ECP, S-EPO, and U-EPX/EDN in the group in which no symptoms developed after withdrawal, indicating that low levels can help to estimate the short-term prognosis and the need for corticosteroid treatment over a limited time. Corresponding correlations could not be found in the continuous treatment group. One reason for this difference could be that exacerbation appeared later in the continuous treatment group, and thus baseline parameters were not as closely related to the late relapses. We found a significant correlation between the number of PBEs in blood, S-ECP, and S-EPO. However, S-ECP or S-EPO had no advantages compared with PBE count in predicting exacerbation and airway inflammation in this group of children. There are some advantages to measuring eosinophils in blood rather than S-ECP and S-EPO. The eosinophils are easily accessible from a venous or a capillary vessel, and the assay can be run routinely. ECP measurements in serum require carefully standardized collection procedures. Interestingly, it has recently been reported that ECP is probably stable in whole blood samples for 24 hours, when stored at 4°C after the standardized coagulation procedure.²³ If this can be confirmed in a larger scale study, the clinical usefulness of the ECP analysis may improve.

All eosinophil markers were significantly lower in the healthy children, and we therefore propose that any one of these markers may help us to distinguish healthy children from children with airway inflammation when the diagnosis is unclear as a result of vague or atypical symptoms, but this topic deserves further study. EPX in urine, if measured at a standardized time point, could be the preferred marker for this purpose because of the easy sampling procedure.

Previous prospective studies of cessation of corticosteroid therapy have assessed deterioration in asthma by means of lung function, BHR measurements, and symptoms.^{13,14,24} In a pediatric study airway responsiveness, FEV_{10} , and PEF deteriorated in children within 1 month after treatment with 600 μg budesonide tapered off.²⁴

These results contrast with the findings of Juniper et al,¹⁴ who reported stable airway responsiveness but decreased FEV₁ and clinical symptoms, in adult patients after 3 months without inhaled budesonide at 400 µg/d. In our study we could not demonstrate any consistent deterioration in BHR in the withdrawal group within 4 months after cessation of low doses of budesonide. This could be explained by fairly mild disease in our children and/or by too short an observation time. Our results might also have been affected by missing BHR results from the 3 children in the withdrawal group who had to be excluded from the study because of exacerbation of asthma.

We did not find any correlation between lung function and markers of inflammation in peripheral blood, as in the study by Wilson et al,²⁵ indicating that there is no strict relationship between lung function and airway inflammation assessed in peripheral blood. This lack of relationship may reflect different mechanisms underlying the asthma disease.⁷

Withdrawal studies in adults have reported increased numbers of PBEs in combination with increased exacerbation rates of asthma.^{26,27} In agreement with that, we found—on a group level—a correlation between increasing numbers of PBEs and serum levels of ECP and EPO proteins and an increasing risk of asthma exacerbation. However, it is necessary to differentiate between results on a group level and results in individual patients. We found that in individual patients, low (normal) numbers/levels could be used to predict the course of asthma because low numbers/levels correlated with a low risk of exacerbation up to 4 months after withdrawal of inhaled budesonide in these children with allergic asthma.

Until more sensitive, carefully evaluated markers of inflammation become easy to use, we suggest that measurement of eosinophil count in blood could provide information about the risk of exacerbation in allergic asthma and help to estimate the degree of airway inflammation.

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