Adolescents in Clinical Remission of Atopic Asthma Have Elevated Exhaled Nitric Oxide Levels and Bronchial Hyperresponsiveness

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Symptoms of atopic asthma often decrease or even seem to disappear around puberty. The aim of this study was to investigate whether this so-called clinical remission is accompanied by remission of airway inflammation, since symptoms relapse in a substantial proportion of subjects later in life. To assess indicators of inflammation and/or structural damage of the airways, exhaled nitric oxide (eNO) and bronchial responsiveness to adenosine-5′-monophosphate (AMP) and methacholine (MCh) were determined in 21 subjects in clinical remission of atopic asthma. Clinical remission was defined as complete absence of symptoms of asthma without the use of any medication in the year preceding the study. Results were compared with those of 21 patients with current asthma and 18 healthy control subjects. We found significantly higher eNO values in the remission group than in healthy controls (geometric mean, 18.9 and 1.0 ppb, respectively; $p < 0.001$) whereas eNO values of the remission group and those of the subjects with current asthma (geometric mean, 21.9 ppb) were similar ($p = 0.09$). The responsiveness to both AMP and MCh of subjects in clinical remission was significantly higher as compared with responsiveness of healthy controls, and lower than responsiveness of subjects with current asthma. A significant correlation could be established between eNO and responsiveness to AMP, but not between eNO and responsiveness to MCh. The results of this study are suggestive of persistent airway inflammation during clinical remission of atopic asthma. We speculate that subclinical inflammation is a risk factor for asthma relapse later in life, and that eNO and responsiveness to both AMP and MCh can be used as different, noninvasive indices of the inflammatory process of the airways.

Asthma is a chronic inflammatory disorder of the airways, characterized by cellular infiltration, cellular activity, and cell damage, but also by edema, vascular leakage, and hypertrophy/hyperplasia of resident cells, as there are goblet cells and smooth muscles. Structural changes of the airway walls occur early in the course of the disease (1, 2). Epidemiological studies have shown that symptoms of atopic asthma often begin in early childhood and improve or seem to disappear around puberty (3, 4). However, a considerable proportion of individuals with asthma in "clinical remission" will have a relapse later in life (3). Several studies have shown spirometric abnormalities and bronchial hyperresponsiveness (BHR) to methacholine (MCh) or cold air challenge during clinical remission of asthma (5, 6). It is unknown whether these functional abnormalities, which are supposed to be indicative of asthma severity with respect to symptomatic asthma, reflect persistent airway inflammation or merely indicate residual airway damage. These considerations seem to be important, as it is reasonable to believe that persistent airway inflammation during clinical remission of atopic asthma has substantial impact on the risk of relapse at a later age. The question arises whether other available noninvasive physiological techniques can give information about the presence of an ongoing inflammatory process.

Nitric oxide synthase (NOS) is a newly identified enzyme system active in airway epithelial and endothelial cells, macrophages, neutrophils, mast cells, autonomic neurons, smooth muscle cells, and fibroblasts. The existence of an inducible form of NOS (iNOS) in human lungs suggests that increased production of NO, probably induced by cytokines, may be relevant to the pathology of asthma (7). Many studies concerning atopic asthma demonstrate elevated NO levels in exhaled air (8, 9). Furthermore, exhaled NO (eNO) levels are decreased by antiinflammatory therapy, which offers opportunities to monitor compliance with and effectiveness of treatment (10). The measurement of eNO can be performed repeatedly, even in children and patients with severe airflow obstruction, in whom invasive techniques are not feasible or desirable. Studies regarding eNO reported a weak relationship between eNO levels and BHR (11, 12), indicating the complexity of mechanisms involved in atopic asthma. The usefulness and specificity of eNO values with respect to the monitoring of airway inflammation in atopic asthma are still under investigation.

BHR is one of the hallmarks of asthma and is often used as an indicator of asthma severity. Several studies have shown a significant correlation between BHR and airway inflammation in symptomatic asthma (13–15). The degree of airway responsiveness can be assessed with a variety of inhaled stimuli, such as MCh or adenosine-5′-monophosphate (AMP). MCh induces airway constriction via direct stimulation of the muscarin receptors on airway smooth muscle cells. AMP, on the other hand, causes airway narrowing mainly through indirect mechanisms, in particular stimulation of mast cells and activation of neuronal reflexes in the lung (13). Since mast cells are believed to play a predominant role in atopic asthma, the bronchial response to AMP, in addition to the response to MCh, may serve as indicator of the inflammatory process.

The aim of this study was to measure eNO values and bronchial responsiveness to MCh and AMP, each reflecting distinct parts of the inflammatory process in the airways, in previously well-documented atopic asthmatic adolescents who were in clinical remission for more than one year. Data were compared with data on adolescents with current asthma and healthy control subjects.

METHODS

Subjects

Adolescents, 18–25 yr of age, with atopic asthma were selected from the Sophia Children's Hospital (Rotterdam, The Netherlands) dis-

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charged patient files. Clinical remission was assumed if subjects reported complete absence of cough, wheezing, and breathlessness at rest and on exertion and had not taken any medication in order to control asthmatic symptoms for at least 12 mo before the study. Twenty-one eligible subjects in remission were included, and compared with 21 patients with asthma who had persistent symptoms at least once a month in the year preceding the study, and used inhaled β_2-agonists on demand in order to relieve symptoms. All subjects had a history of wheezing and chest tightness and were previously diagnosed as having atopic asthma according to American Thoracic Society (ATS) criteria (16). In the past, all had a provocation dose/concentration of MCh or histamine producing a 20% fall in FEV_1 of $\leq$ 150 μg (dosimeter method) or $\leq$ 8 mg ml^{-1} (2-min tidal breathing method), and/or had an FEV_1 reversibility $\geq$ 12% of predicted normal value. All had evidence of atopy defined by radioallergosorbent test (RAST) Class 2 or higher for at least one common airborne allergen. Participating subjects were lifelong nonsmokers, in stable clinical condition, and did not take inhaled steroids, including nasal steroids, or antiallergic medication such as cromoglycate and antihistamines for at least 1 yr before the study.

Eighteen healthy nonsmoking young adult volunteers were recruited via advertisement and served as control subjects. They had a negative personal and first-degree family history of asthma and atopy. Common exclusion criteria were an inability to perform lung function tests reproducibly, perennial rhinitis, and other illnesses that may affect lung function. None of the subjects in the study reported symptoms of respiratory infection in the month before the study. The study was approved by the Medical Ethics Committee of the Erasmus University and University Hospital Rotterdam.

Study Design

We performed a cross-sectional study with three visits on separate days. At the first visit, subjects gave informed written consent and were asked about asthmatic symptoms and requirement for rescue medication during the past year, especially to avoid the inclusion of subjects with mild symptoms of asthma in the remission group. For the same reason, subjects were asked to complete Juniper's Quality of Life Questionnaire (17), which was verified by the investigator on hidden minor symptoms. Also, physical examination was performed and eNO and PD_{20}MCh (provocative concentration of MCh causing a 20% fall in FEV_1) determined. At Visit 2, scheduled at least 1 d after Visit 1, subjects underwent an AMP challenge test. At Visit 3, scheduled at least 3 d after Visit 2, FEV_1 and FEV_1 reversibility were tested. The sequence and intervals were chosen to avoid any influence of the AMP challenge on MCh responsiveness and/or on FEV_1 (18). The maximal time allowed between Visits 1 and 3 was 3 wk.

Spirometry

Short acting β_2-agonists were not allowed within 8 h before the test. Flow–volume curves were obtained with a Lilly-type pneumotachograph (Masterlab Jaeger, Würzburg, Germany). The best of three reproducible recordings of FEV_1 was expressed as percentage of predicted normal and used for analysis. Reversibility was tested by measuring FEV_1 before and 20 min after inhalation of 1 mg of terbutaline powder (Bricanyl Turbuhaler; Astra, Lund, Sweden), and expressed as increase in percentage of predicted normal.

Nitric Oxide

On the basis of the recommendations of the European Respiratory Society Task Force (19), eNO was measured in exhaled air by means of chemiluminescence (model 280 nitric oxide analyzer; Sievers, Boulder, CO) with a sensitivity of 0.1 ppb and a detection range of $<$ 0.1–500,000 ppb. The sampling flow was 0.2 L/min and the response time 0.2 s. Data were displayed continuously on a PC screen, and stored in a computer with a sample frequency of 20 Hz for later analysis. The analyzer was calibrated regularly according to the manufacturer guidelines, employing certified calibration gases containing 0 ppb, 100 ppb, and 9 ppm NO (Hoekloos, Barendrecht, The Netherlands). The measurement circuit consisted of a mouthpiece connected to a two-way nonrebreathing valve (Hans Rudolph, Kansas City, MO), through which subjects inhaled ambient air (if ambient NO was $<$ 10 ppb) or NO-free medical air (if ambient NO was $>$ 10 ppb) while seated, not wearing a noseclip. Subjects inhaled to TLC and immediately exhaled for as long as possible into a tube with an in-line flow resistor (20 cm H_2O L^{-1} s^{-1}; Hans Rudolph). This was done at a flow corresponding to 5% of subject vital capacity per second, with the aid of a visual feedback display. A fine-bore Teflon tube continuously sampled exhaled air from a side port situated directly after the mouthpiece to the analyzer. Water vapor was absorbed by means of an NO-inert filter in the tube. Airflow was measured with a Lilly-type pneumotachograph (Masterlab Jaeger) positioned downstream of the resistor. An end-expiratory plateau of at least 10 s, where flow varied ± 10% of the target flow, was the end point of the measurement. The test was done in triplicate and average eNO at the plateau calculated by means of custom-made software.

Methacholine and Adenosine-5′-Monophosphate Challenge

Challenge tests were performed at the same time of day (± 1 h) according to the dosimeter method validated by Birnie and coworkers (20). Short-acting β_2-agonists were not allowed within 8 h before the test. Calibrated DeVilbiss 646 nebulizers (DeVilbiss Health Care, Somerset, PA) were filled with 3 ml of the appropriate solutions. Subjects inhaled four 5-μl volumes, using a French–Rosenthal dosimeter (Laboratory for Applied Immunology, Fairfax, VA). After recording baseline values, the challenge started with inhalation of 0.9% NaCl. If a patient responded to saline or the lowest concentration of either MCh or AMP, they were assigned a PD_{20} value of half the starting dose. Inhalation provocation tests were performed with doubling concentrations of 0.15 to 78.4-mg/ml MCh bromide in phosphate-buffered saline (PBS) or 0.08- to 160-mg/ml AMP in normal saline (Sigma, St. Louis, MO). Provocative doses causing a 20% fall in FEV_1 (PD_{20}) from baseline were calculated by means of linear interpolation of the logarithmic dose–response curve. If FEV_1 fell less than 20% of the prechallenge level at the highest dose administered, twice the highest dose was arbitrarily used as the PD_{20} value. An MCh PD_{20} value of 1,000 μg, corresponding approximately to 7.8 μmol cumulative, was considered the cutoff value for bronchial hyperresponsiveness to MCh.

Peak Expiratory Flow Rate

After being instructed by the investigator at Visit 1, the patients recorded their peak expiratory flow rate (PEFR) with a Personal Best peakflow meter (Respironics, Nantes, France) twice daily at home during the period between Visit 1 and Visit 3. PEFR was always recorded before bronchodilatation. Each measurement consisted of three attempts, of which the highest value was used for further analysis.

Statistical Analysis

Because of their highly skewed distributions, PD_{20} and eNO values were analyzed after logarithmic transformation. Mean data were expressed as geometric mean $e^{\pm SEM(\ln X)}$. With respect to PD_{20} values, comparisons between groups were made by the Mann–Whitney test for unpaired samples. Correlation between different tests was made by the Spearman rank correlation test. The distributions of all other variables were not significantly different from a standard normal distribution. Hence, parametric techniques (Student *t* test, Pearson correlation coefficients) were applied. Mean values of these parameters were expressed as means ± SEM. A two-tailed p value of less than 0.05 was considered significant. Data were analyzed with the Statistical Package for the Social Science (SPSS, Chicago, IL) for Windows version 8.0.

RESULTS

Sixty subjects completed the study (21 subjects with current asthma, 21 subjects in clinical remission, and 18 healthy controls). The male-to-female ratio was 15 to 6 in the asthmatic group, 18 to 3 in the remission group, and 10 to 8 in the control group. Age did not differ significantly between the groups (22 ± 2 yr in the asthmatic group, 21 ± 2 yr in the remission group, and 24 ± 1 yr in the control group). Duration of remission in the remission group varied from 1 to 12 yr (median, 5 yr). All subjects of the asthma group and the remission group, and

TABLE 1

EXHALED NO AND LUNG FUNCTION IN SUBJECTS WITH AND WITHOUT CLINICAL REMISSION OF ASTHMA, AND IN HEALTHY CONTROL SUBJECTS

Parameter*	Subjects with Asthma (*n* = 21)	Subjects in Clinical Remission of Asthma (*n* = 21)	Healthy Control Subjects (*n* = 18)
eNO, ppb	$22e^{\pm 0.19\dagger}$	$14e^{\pm 0.15\dagger}$	$1e^{\pm 0.31}$
PD_{20}MCh, μg	$94e^{\pm 0.37\dagger}$	$752e^{\pm 0.31\ddagger}$	$4954e^{\pm 0}$
PD_{20}AMP, μg	$1110e^{\pm 0.37\dagger}$	$5704e^{\pm 0.22\S}$	$10496e^{\pm 0}$
FEV_1 % pred	$88 \pm 12^{\dagger}$	$93 \pm 15^{\|}$	105 ± 13
Reversibility FEV_1, %	$11 \pm 1^{\dagger}$	$7 \pm 1^{\#}$	4 ± 1
Diurnal PEFR variation, %	13 ± 2	11 ± 1	8 ± 1

Definition of abbreviations: PD_{20}MCh = provocative dose of methacholine causing a 20% fall in FEV_1. PD_{20}AMP = provocative dose of adenosine-5′-monophosphate causing a 20% fall in FEV_1; reversibility FEV_1 = change in FEV_1, expressed as increase in percentage of predicted normal value after administration of 1 mg of terbutaline.

* eNO, PD_{20}MCh, and PD_{20}AMP are expressed as geometric mean $e^{\pm SEM(\ln X)}$. All other variables are expressed as means ± SEM.

† $p < 0.001$ versus healthy controls.

‡ $p < 0.001$ versus healthy controls and asthmatics.

§ $p < 0.01$ versus healthy controls and asthmatics.

‖ $p = 0.02$ versus healthy controls.

# $p < 0.02$ versus healthy controls and asthmatics.

two subjects of the control group, had positive RAST tests. Results of eNO measurement, bronchial provocation tests, and spirometry are summarized in Table 1.

Exhaled NO Values

Geometric mean eNO values in the remission group were significantly higher than in healthy controls (mean, 18.9 and 1.0 ppb, respectively; $p < 0.001$), and slightly but not significantly lower than those of subjects with asthma (21.9 ppb; $p = 0.09$) (Table 1; Figure 1). Although there seemed to be a trend toward lower eNO values with longer duration of remission, a significant correlation between these variables could not be established.

Bronchial Challenge Tests

In the remission group, 11 of 21 subjects had a PD_{20}MCh of less than 1,000 μg, compared with none of the control subjects. Of the subjects with asthma, 19 of 21 showed a PD_{20}MCh value below this level. PD_{20}MCh values in the remission group were significantly lower than in the control group (geometric mean, 751 and 4,954 μg, respectively; $p < 0.001$), but higher than in the asthmatic group (94 μg; $p < 0.001$) (Figure 2). The AMP challenge showed significantly lower PD_{20}AMP values in the remission group than in the control group (geometric mean, 5,704 and 10,496 μg, respectively; $p < 0.01$), but higher values in the remission group than in the asthmatic group (1,110 μg, $p < 0.01$) (Figure 3). In subjects in remission and those with asthma, PD_{20}MCh and PD_{20}AMP were highly correlated ($r = 0.72$, $p < 0.001$). There was a significant inverse correlation between PD_{20}AMP and eNO ($r = -0.51$; $p = 0.001$), while no correlation could be established between PD_{20}MCh and eNO ($r = -0.25$; $p = 0.1$). There was no significant relationship between formerly and currently assessed bronchial responsiveness, or between duration of remission and bronchial responsiveness.

Spirometry and PEFR

Baseline FEV_1 values, expressed as percentage of predicted normal value, were significantly reduced in subjects in remission compared with healthy control subjects (mean, 93 and 105%, respectively; $p = 0.02$). No significant difference could be detected between subjects in remission and subjects with asthma (mean, 88%). Reversibility in FEV_1 was significantly different ($p < 0.02$) between the three groups (mean, 11, 7, and 4% in subjects with asthma, subjects in remission, and healthy control subjects, respectively). Mean diurnal PEFR variation was 13% in subjects with asthma, 11% in subjects in

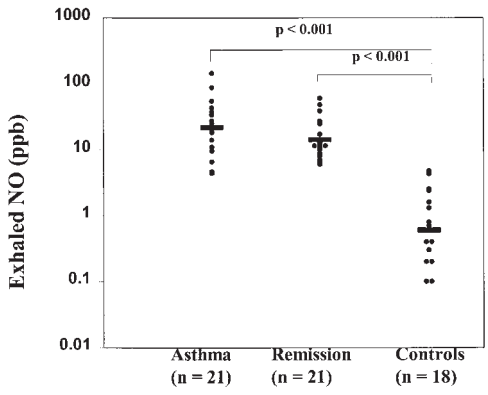

Figure 1. Exhaled NO values of currently asthmatic subjects, of subjects in clinical remission of atopic asthma, and of healthy controls. Each dot represents one subject. *Horizontal bars* represent geometric mean values. The *y* axis shows NO values logarithmically.

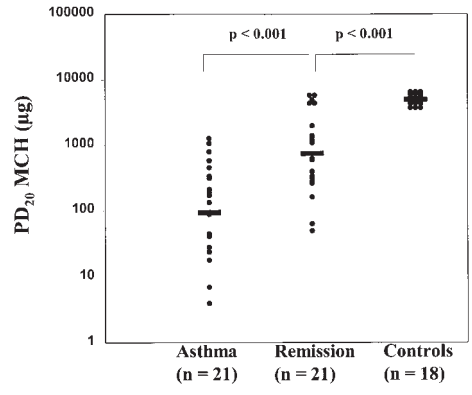

Figure 2. PD_{20}MCh (micrograms, not cumulative) values of currently asthmatic subjects, of subjects in clinical remission of atopic asthma, and of healthy controls. Nonresponders are arbitrarily given a value of twice the highest dose administered. *Horizontal bars* represent geometric mean values. The *y* axis shows PD_{20}MCh values logarithmically.

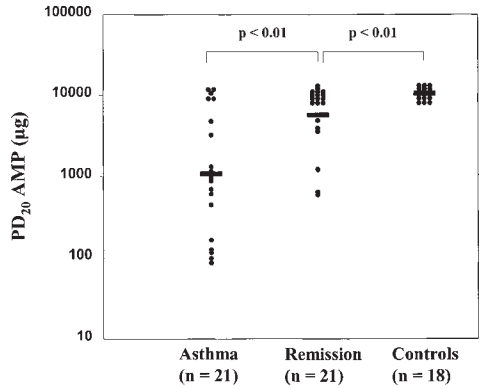

Figure 3. PD_{20}AMP (micrograms, not cumulative) values of currently asthmatic subjects, of subjects in clinical remission of atopic asthma, and of healthy controls. Nonresponders are arbitrarily given a value of twice the highest dose administered. *Horizontal bars* represent geometric mean values. The *y* axis shows PD_{20}AMP values logarithmically.

remission, and 8% in control subjects, and showed no significant difference between the groups.

DISCUSSION

In this study, we demonstrated elevated levels of eNO and airway hyperresponsiveness to MCh and AMP in adolescents in long-standing clinical remission of atopic asthma. During remission, eNO levels were almost similar to those found in subjects with current asthma. There was a significant correlation between eNO values and AMP responsiveness, but not between eNO values and MCh responsiveness. A significant relationship between duration of remission and degree of abnormalities could not be established.

Studies regarding completely symptom-free subjects who had well-documented atopic asthma in the past are scarce (5). In our study, subjects were regarded to be in clinical remission when they reported complete absence of symptoms and were without treatment for at least 1 yr preceding the study. This definition was applied to avoid the inclusion of asthmatic subjects with mild symptoms in the remission group. The possibility that subjects with mild symptoms were included in other studies on asthma remission cannot be ruled out. In one of these studies, asthma was considered inactive when subjects reported no asthma attacks or use of medication during the preceding year, and were without frequent episodes of shortness of breath with wheezing (6). Following this definition of remission, the investigators reported bronchial hyperresponsiveness to MCh with or without airflow obstruction in subjects aged 18–61 yr in remission.

Despite the relatively long median duration of remission, we found that subjects in clinical remission had significantly elevated eNO levels, as compared with eNO levels from healthy controls. Exhaled NO originates from several inflammatory cell types in the airways, including epithelial and endothelial cells and macrophages. Whether elevated eNO levels are caused by enhanced activity of inflammatory cells expressing NOS, potentially driven by inflammatory mediators or cytokines, and/or by enhanced diffusion through the airway wall due to structural damage, remains to be resolved. An increasing number of articles shows evidence that eNO is related to atopic asthma more than to nonatopic asthma or atopy per se. It has been demonstrated that atopic asthmatic children had higher geometric mean eNO levels than did nonatopic asthmatic children, atopic nonasthmatic children, or nonatopic nonasthmatic children, suggesting that both atopy and asthma are important in the context of elevated eNO levels (21). In other studies, eNO levels were significantly higher in atopic subjects with asthma compared with levels from nonatopic asthmatic subjects (22, 23). Similar results were obtained in patients with rhinitis, whereas no difference was found in eNO levels between atopic and nonatopic control subjects (22). Horvath and Barnes found elevated eNO levels in atopic asymptomatic subjects, but suggested that this finding merely reflected an early stage of airway inflammation, possibly preceding the onset of asthmatic symptoms (24). In our study, two subjects in the control group had a positive RAST test, but had no elevated eNO values. Thus, with reference to the suggestion of Horvath and Barnes, the presence of elevated eNO levels in our atopic remission group might well be due to subclinical airway inflammation. This is also supported by other studies in which a positive correlation was reported between eNO levels and markers of eosinophilic airway inflammation in induced sputum (9, 25). Others demonstrated an association between eNO and exposure to relevant allergens (26), or a reduction in eNO after antiinflammatory treatment (27). These study results provide sufficient indications that airway inflammation is significantly associated with elevated eNO levels, thus providing a tool in monitoring disease activity. However, a range of normality needs to be established.

The eNO levels measured in this study tend to be somewhat lower than those measured in other studies (9, 28). This may be caused by technical factors. It has been shown that there is a marked flow dependence of eNO values, with lower values measured at high flow rates and vice versa (29). We standardized the expiratory flow as 5% of vital capacity per second, which resulted in an expiratory flow of approximately 0.25 to 0.30 ml/min, which is higher than the flow used in some other studies. It is not clear whether such standardization of flow for lung volume is important when measuring eNO. It has been demonstrated that the flow–eNO relationship differed between subjects with asthma and healthy individuals (30). This difference is, however, of minor importance compared with the differences we now report between the various groups.

We believe that responsiveness to AMP may serve as an indicator of airway inflammation, although normal values for bronchial AMP responses have not been determined yet. It was demonstrated a few years ago that experimental instillation of AMP into an airway segment caused a prompt reduction in airway caliber, paralleled by a significant rise in prostaglandin D_2, histamine, and tryptase levels in the lavage fluid, suggestive of mast cell degranulation (31). Since mast cells are believed to play a predominant role in asthmatic airway inflammation, the response to AMP may reflect different aspects of inflammation as compared with the bronchial response to direct stimuli, such as methacholine or histamine. Results supporting this statement include a more pronounced improvement of AMP responsiveness after avoidance of allergen (32), and after antiinflammatory therapy (33), as compared with effect on MCh or histamine responsiveness. It has been suggested that an exaggerated airway response to direct stimuli, such as MCh, and abnormal FEV_1 values, may exist or persist independently of "active" airway inflammation (34). It is probable that these indices are also influenced by irreversible airway damage, caused by airway remodeling (35). Thus, being associated with the more slowly responding elements of inflammation within the airways. Our finding of a positive correlation between response to AMP and eNO levels, but not between responsiveness to MCh and eNO levels, is in agreement with this.

The clinical relevance of our findings is as yet unknown. Although one would expect the degree of abnormalities to be related to the duration of remission, a significant correlation could not be established. Probably, when subclinical airway inflammation is present during adolescence, it may persist for several years with a continuous risk of becoming clinically manifest again. Future longitudinal studies should assess the possible benefits of prolonged disease monitoring and antiinflammatory treatment during the asymptomatic phase. Also, the question arises concerning whether indices associated with different parts of the inflammatory process, such as eNO and responsiveness to AMP and MCh, need to be included in the definition of asthma remission.

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