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Bronchial hyper-responsiveness to hypertonic saline and blood eosinophilic markers in 8–13-year-old schoolchildren

G. de Meer*, D. S. Postma†, N. A. H. Janssen*, J. C. de Jongste‡ and B. Brunekreef*

*Institute for Risk Assessment Sciences, Environmental & Occupational Health, Utrecht University, The Netherlands, †Pulmonology, University Hospital, Groningen, The Netherlands and ‡Pediatric Pulmonology, Erasmus Medical Center, Rotterdam, The Netherlands

Summary

Background In adult asthma, bronchial hyper-responsiveness (BHR) to indirect stimuli reflects eosinophilic activation more closely than BHR to stimuli that directly cause smooth muscle contraction.

Aim To assess the relationship between BHR to the indirect stimulus hypertonic saline (HS), blood eosinophil numbers, and serum eosinophilic cationic protein (ECP) in children with and without current wheeze.

Methods A cross-sectional survey among 8–13-year-old schoolchildren, using the International Study of Asthma and Allergic disease in Childhood questionnaire, bronchial challenge with HS, skin prick tests, serum IgE, blood eosinophil counts and ECP (in a subset). Based upon the presence of current wheeze (WHE) and BHR, we defined three case groups (WHE+BHR+, WHE − BHR+, WHE+BHR −) and the reference group (WHE − BHR −). By regression analyses, each case group was compared with the reference group for differences in atopic sensitization, blood eosinophil counts and serum ECP.

Results Complete data were obtained for 470 children. BHR was present in 103 children (22%), 66 being asymptomatic and 37 symptomatic. Children of all three case groups were more often atopic. Sensitization to indoor allergens particularly occurred in children with BHR, irrespective of symptoms ($P<0.05$).

Children with WHE+BHR+ had highest values for blood eosinophils and serum ECP ($P<0.05$). Children with WHE − BHR+ had less severe responsiveness. In atopic children with WHE − BHR+, serum ECP was higher than in children with WHE-BHR-($P<0.05$).

Conclusions BHR to HS is associated with blood markers of eosinophilic activation, particularly in atopic children.

Keywords asymptomatic BHR, atopy, blood eosinophils, serum ECP, symptomatic BHR
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Introduction

Asthma is characterized by variable airway obstruction and bronchial hyper-responsiveness (BHR). Chronic inflammation is presumed to be the principal pathology, with remodelling of the airways as the final outcome. Eosinophils and mast cells constitute the predominant cell types in the inflammatory process [1–4]. Mast cell mediators stimulate the release mediators from eosinophils [5–7], and conversely eosinophilic mediators can activate mast cells [8, 9]. This inflammatory process is thought to contribute to the presence of airway hyperresponsiveness, a core feature of asthma.

In epidemiological studies, BHR in children is commonly assessed by inhalation of hypertonic saline (HS). The mechanism of airway constriction in response to this stimulus acts by increasing airway osmolarity, which is presumed to cause degranulation of mediators from mast cells such as histamine and leukotrienes that subsequently cause bronchoconstriction [10]. The increased number and activation state of mast cells in asthmatic subjects explains the higher sensitivity of asthmatics to inhaled HS. The observed relationship between mast cells and eosinophils furthermore suggests that BHR to HS may be a marker of eosinophilic inflammation in the bronchi. This is supported by observations that corticosteroid treatment reduces BHR to HS to a greater extent than BHR to methacholine [11–13]. More directly, Gibson et al. [14] found a dose–response relationship between the dose of HS causing a 15% fall in FEV_1 (PD_{15}) and sputum eosinophil numbers, whereas no such relationship was observed for BHR to methacholine. In a general population study, BHR to HS was also associated with a higher number of sputum eosinophils, though only if symptoms were present as well [15]. However, this population comprised only 156 children of whom 23 (15%) had BHR, and limited subgroup analysis.

The aim of the present study was to evaluate eosinophilic activation, assessed by blood eosinophil counting and serum

Correspondence: Gea de Meer, Institute for Risk Assessment Sciences, Environmental & Occupational Health, Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands. E-mail: G.demeer@iras.uu.nl

eosinophilic cationic proterin (ECP), in BHR to HS. We hypothesized that such a relationship would be independent of the presence of wheeze in the past 12 months.

Methods

Study population and design

The population comprised 2207 Dutch schoolchildren, aged 8–13 years, who participated in a cross-sectional study on respiratory health effects of living close to freeways. Respiratory health was assessed using International Study of Asthma and Allergic disease in Childhood (ISAAC) phase 2 standards, including questionnaire data on asthma symptoms ($N = 2159$), skin prick tests (SPTs) ($N = 1350$) and bronchial challenge test with HS ($N = 1258$) [16]. In addition, we included blood withdrawal ($N = 1073$) for eosinophil counting ($N = 1030$), and IgE analyses ($N = 1036$). In 521 children serum eosinophilic cationic protein (ECP) was assessed by a 1 : 2 nested case–control sample in which individuals with an affirmative answer on 'ever asthma', 'wheeze in the past 12 months' or 'dry cough without a cold in the past 12 months', were considered cases.

The Medical Ethical Board of the University of Wageningen approved the study and written informed consent was obtained for each child from a parent or legal locum. Parents had to give permission for each test separately, which explains the difference in participation for the various parts of the study. The final study population for this analysis constituted of 470 children who participated in all tests.

Questionnaire

In addition to the ISAAC questionnaire, we collected data on socio-demographic characteristics, housing conditions, medical treatment, family history of asthma, and environmental tobacco smoke (ETS).

Atopic status

Atopy was assessed by SPT and serum-specific IgE. SPTs were performed according to the ISAAC phase 2 standards to a panel of seven common allergens (ALK-Abello, Horsholm, Denmark). Serum-specific IgE was assessed by the CAP-assay (Pharmacia, Woerden, the Netherlands). The Phadiatop was used as a screening instrument for allergy to common inhalant allergens [17]. Sera with a positive result were tested for specific IgE. Allergens tested in SPTs and/or IgE analysis comprised: (1) mixed grass pollen (*Anthoxanthum odoratum*, *Avena eliator*, *Dactylis glomerata*, *Festuca pratensis*, *Holcus lanatus*, *Lolium perennae*, *Phleum pratense*, *Poa pratensis*, *Secale cereale*), (2) mixed tree pollen (*Alnus glutinosa*, *Betula verrucosa*, *Corylus avellana*, *Quercus alba*, *Salix caprea*), (3) cat dander, (4) dog dander, house dust mites (HDMs), (5) *Dermatophagoides farinae*, (6) *D. pteronyssinus*, and (7) moulds (*Alternaria tenuis*, *Cladosporium herbarum*, *Penicillinum notatum*).

A positive SPT was defined if the mean weal diameter $\geqslant 3$ mm, and if result on specific IgE as a titre of $\geqslant 0.35$ kU/L is positive. Children with either a positive result by SPT or by specific IgE were considered atopic. We distinguished indoor allergens as HDM, cat and dog dander, and outdoor allergens as moulds, grass and tree pollen.

Serum ECP

Serum ECP was determined by fluoro-immunoassay (Pharmacia) by the same lab that performed IgE analyses (Pharmacia). Blood and serum handling occurred according to the test manufacturer's protocol.

Blood eosinophils

Blood eosinophils were determined by Coulter counter autoanalyser and expressed as the number of cells per litre (AML, Anvers, Belgium).

Lung function and bronchial challenge

Prior to the bronchial challenge test, lung function was determined using a pneumotachometer (Jaeger, Würzburg, Germany) according to guidelines of the ERS [18]. Salbutamol was stopped 6 h before the bronchial challenge test, antihistaminics and cromoglycate 48 h. Children were excluded from bronchial challenge if the forced expiratory volume in 1 s (FEV_1) was below 75% of the predicted value ($N = 0$), or if they were unable to perform acceptable forced spirometry manoeuvre at baseline ($N = 17$). Data of another 12 children were excluded because they were unable to complete the test because of excessive cough.

Briefly, an aerosol of 4.5% saline was inhaled for 0.5, 1, 2, 4 and 8 min, and two reproducible measurements of FEV_1 were achieved 1 min after each inhalation step of which the higher was selected. The test stopped after completing all inhalation steps, or if there was a fall in $FEV_1 \geqslant 15\%$ compared with the highest pre-challenge FEV_1. PD_{15} HS was assessed by linear interpolation and BHR was defined as a PD_{15} HS $\leqslant 23$ mL, which has shown similar sensitivity and specificity for asthma symptoms and diagnosis as BHR to histamine [19]. To include a measure of bronchial responsiveness for each child, including those without BHR, we calculated the dose–response slope (DRS) as the maximum % fall in FEV_1 per inhaled millitre of HS [20, 21].

Statistical analysis

Logistic regression analysis was performed to select respiratory symptoms that were independently associated with BHR. To this, we evaluated symptoms in the past 12 months of wheeze, a dry cough, chronic phlegm, and a doctor's diagnosed bronchitis. In bivariate analyses current wheeze and a dry cough were associated with BHR (odds ratio $(OR)_{wheeze} = 3.46$, 95% CI 2.48; 4.83, and $OR_{cough} = 1.77$, 95% CI 1.27; 2.46). In multivariate analyses with both symptoms in the model, statistical significance was only present for wheeze (OR 3.43, 95% CI 2.39; 4.91). Therefore, we defined outcomes on asthma symptoms and BHR by the presence of wheeze in the past 12 months as

WHE+BHR+ 'Wheeze in the past 12 months' *and* BHR
WHE − BHR+ No 'wheeze in the past 12 months' *and* BHR
WHE+BHR − 'Wheeze in the past 12 months' *and* no BHR
WHE − BHR − No 'wheeze in the past 12 months' *and* no BHR

WHE, wheeze; BHR, bronchial hyper-responsiveness.

For each case group (WHE+BHR+, WHE − BHR+, WHE+BHR −), the differences in atopy, blood eosinophils, serum ECP, and DRS were assessed using children without wheeze and no BHR (WHE − BHR −) as reference group. Regression analysis was performed in order to adjust for potential confounders: logistic regression for atopic sensitization, and linear regression for continuous variables (DRS, blood eosinophils, serum ECP). Prior to linear regression, variables were log transformed to normalize the distributions. The results were expressed as ORs for logistic regression, and percentage difference compared with the reference for linear regression. Since eosinophils and ECP are associated with atopy irrespective of the presence of asthma, we repeated the analyses for atopics only.

All statistical analyses were performed using the SAS 8.02 statistical software package.

Results

The study population comprised 470 children who completed all tests. Table 1 shows characteristics of participants and non-participants. Participants were older, more often boys, and more often atopic. The prevalence of wheeze, BHR, and a parental history of atopy were similar in participants and non-participants.

As shown in Table 2, BHR was present in 103 children, 37 (36%) symptomatic. These 37 children comprised 43% of the 86 children with recent wheeze. In children with BHR, the degree of responsiveness was higher if symptoms were present as well, irrespective whether expressed as DRS or PD_{15}. In children without BHR, the DRS was similar in children with and without symptoms.

Atopy occurred more often in children with BHR and/or symptoms compared with children without symptoms and no BHR (WHE − BHR −). The higher sensitization rate was most pronounced for indoor allergens. Particularly HDM sensitization occurred more frequently in all three outcomes with wheeze or BHR compared with children without wheeze and no BHR ($P<0.05$). Considering other allergens, children with WHE+BHR+ consistently had highest sensitization rates (all $P<0.05$ compared with WHE − BHR −). Adjustment for potential confounders by logistic regression analyses yielded similar results (Table 3), except for a loss of statistical significance for sensitization to indoor allergens in children classified as WHE+BHR − . Lack of statistical power due to small numbers limited more detailed analyses on the contribution of individual allergens.

Figure 1 shows the results of multiple regression analyses for blood eosinophils and serum ECP. Blood eosinophils and serum ECP were higher in children with symptomatic BHR (Fig. 1a). Considering atopic children only (Fig. 1b), differences with the reference group of WHE − BHR − became more pronounced. Again blood eosinophils and serum ECP were highest in children with symptomatic BHR ($P<0.05$). In addition, atopic children with asymptomatic BHR had a higher level of serum ECP ($P<0.05$). DRS and PD_{15} were similar in atopic and non-atopic children with asymptomatic BHR.

In the analyses above, we used wheeze in the past 12 months as discriminatory symptom since it was the only symptom associated with BHR in logistic regression analyses that included other respiratory symptoms as well. Bias might occur if other asthma symptoms are present in children categorized as asymptomatic. For 'a dry cough at night in the past 12 months', this occurred in 21% and 42% of the children with WHE − BHR+ and WHE − BHR − , respectively. Exclusion of these children did not change the results.

Corticosteroid treatment is known to reduce asthma symptoms and eosinophilic inflammation and might therefore affect the results. In this population, inhaled corticosteroid

Table 1. Characteristics of participants and non-participants

	Participants ($N=470$)	Non-participants ($N=1737$)
Age, mean (range)	10.4 (8–13)	9.6 (8–13)*
Girls	46	52**
Atopy	37	31**
Wheeze in the past 12 months	18	17
Ever asthma	10	8
Inhaled corticosteroid treatment	4	3
FEV_1, mean %-predicted (range)	100 (75–130)	101 (55–186)
BHR	22	21

*$P<0.01$, **$P<0.05$, for difference between participants and non-participants.
BHR, bronchial hyper-responsiveness; FEV_1, forced expiratory volume in 1 s.

Table 2. Atopic sensitization, pre-challenge lung function, DRS, PD_{15}, blood eosinophils, and serum ECP by outcome defined by the presence of wheeze and BHR

	WHE+BHR+ ($N=37$)	WHE − BHR+ ($N=66$)	WHE+BHR − ($N=49$)	WHE − BHR − ($N=318$)
Atopic sensitization (%)				
Any allergen	81*	44**	47**	29
Indoor allergens†	81*	38**	39**	23
Outdoor allergens‡	60*	26	31	19
FEV_1 (% predicted)§	97 (94, 101)	100 (97, 102)	102 (99, 105)	101 (100, 102)
DRS (% per mL)¶	2.6 (1.9, 3.4)*	1.5 (1.3, 1.8)*	0.2 (0.1, 0.3)	0.2 (0.1, 0.2)
PD_{15} (mL)¶	5.8 (4.3, 7.6)***	9.6 (8.0, 11.4)	Not assessed	Not assessed
Blood eosinophils ($\times 10^6$/mL)¶	347 (269, 446)*	200 (156, 255)	224 (179, 281)	209 (14, 225)
Serum ECP (mg/mL)¶	10.2 (7.2, 14.5)*	6.8 (5.5, 8.3)	7.1 (5.5, 9.2)	6.2 (5.6, 6.8)

*$P<0.01$, **$P<0.05$, compared with WHE − BHR − , ***$P<0.01$ compared with WHE − BHR+. †Moulds, grass or tree pollen. ‡Cat, dog, or HDM (see Methods section). §According to Zapletal et al. [22], mean (95% CI). ¶Geometric mean (95% CI).
DRS, dose–response slope; ECP, eosinophilic cationic protein; BHR, bronchial hyper-responsiveness; WHE, wheeze; FEV_1, forced expiratory volume in 1 s.

Table 3. ORs and 95% CI for atopic sensitization defined by the presence of wheeze and BHR

	Reference: WHE − BHR −		
	WHE+BHR+ OR (95% CI)*	WHE − BHR+ OR (95% CI)*	WHE+BHR − OR (95% CI)*
Atopy			
Any allergen	11.25 (4.38, 28.92)**	2.36 (1.24, 4.50)***	2.11 (1.04, 4.27)‡
Indoor allergens†	12.39 (4.21, 36.50)**	2.34 (1.07, 5.12)***	1.66 (0.69, 3.99)
Outdoor allergens	1.68 (0.65, 4.38)	1.06 (0.46, 2.48)	1.38 (0.56, 3.41)
Number of sensitizations			
Any allergen	2.08 (1.67, 2.59)**	1.25 (1.01, 1.54)***	1.24 (0.99, 1.54)
Indoor allergens	5.49 (3.10, 9.73)**	1.54 (1.00, 2.37)	1.51 (0.97, 2.36)
Outdoor allergens	0.54 (0.24, 1.23)	1.00 (0.56, 1.77)	0.96 (0.51, 1.84)

*Adjusted for age, sex, parental history of asthma or rhinitis, parents' birth country, parental education, current parental smoking, and parent who completed the questionnaire. †Including both atopic sensitization to indoor and outdoor allergens in the model. **$P<0.01$, ***$P<0.05$ compared with WHE − BHR − .
BHR, bronchial hyper-responsiveness; WHE, wheeze; OR, odds ratio; 95% CI, 95% confidence interval.

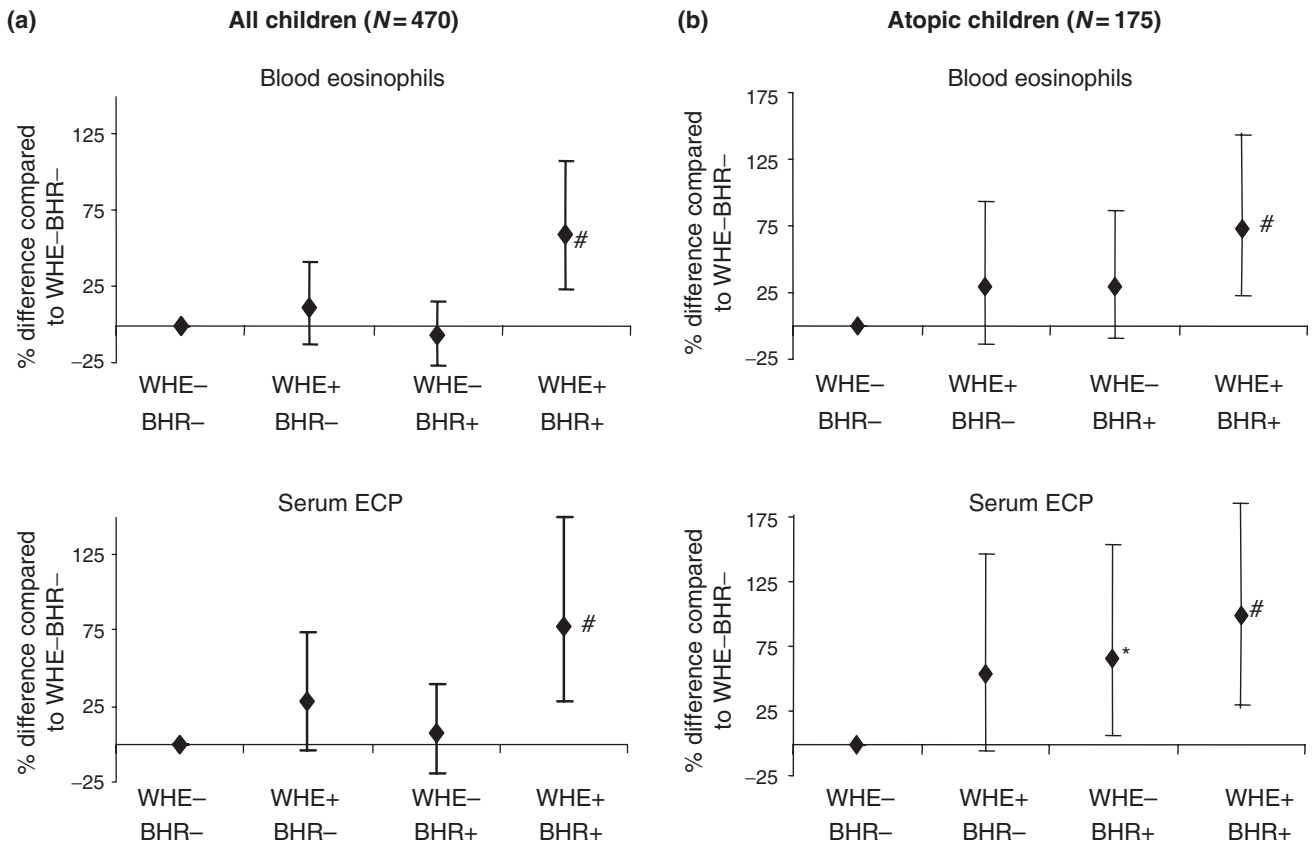

Fig. 1. Blood eosinophils and serum eosinophilic cationic protein (ECP) for different combinations of wheeze (WHE) and bronchial hyper-responsiveness (BHR) in the whole study population (a) and for atopic children (b) separately.

treatment was used in 32% of the children with WHE+BHR+, in 16% with wheeze WHE+BHR − and none of the children with WHE+BHR − or WHE − BHR − . Exclusion of children using corticosteroid treatment did not change the results essentially.

Discussion

In this study among 8–13-year-old schoolchildren, BHR to HS was associated with atopy, particularly to indoor allergens. These associations were independent of the presence of recent wheeze. Children with symptomatic BHR had a higher blood eosinophil number and serum ECP than those without symptoms and/or BHR. In atopic children, serum ECP was also higher in children with asymptomatic BHR compared with children without symptoms and no BHR.

We have made an attempt to estimate the relationship between BHR to HS and eosinophilic inflammation in a general population of schoolchildren. It has been previously shown in atopic asthma that BHR to an indirect stimulus is related to the underlying eosinophilic inflammation, as

assessed in sputum and blood [23]. However, it is largely unknown if such a relationship is present in a general population in which most children do not have a diagnosis of asthma. It is further unknown if eosinophilic inflammation is present in individuals with asymptomatic BHR as well.

Like others, we have found a higher prevalence rate of atopic sensitization in children with BHR, irrespective of the presence of wheeze [24, 25]. Sensitization only involved indoor allergens, and particularly to HDM. In fact, children with symptomatic BHR had the highest sensitization number and rate. Furthermore, bronchial responsiveness was highest in these children. Presumably, children with multiple sensitizations have more severe bronchial inflammation. This may be explained by a greater likelihood to encounter multiple allergens (in frequency and number) that enhance bronchial inflammation.

In this study, we used blood eosinophil counts and serum ECP as proxy markers of eosinophilic activation. Both were increased in symptomatic BHR, but not in asymptomatic BHR. One may argue that blood eosinophilic markers do not properly reflect the action in the airways. Nevertheless, van den Berge et al. [23] found a positive correlation between blood eosinophils and AMP responsiveness, a marker of airway inflammation. However, this study only included subjects with an asthma diagnosis and BHR to methacholine ($PC_{20} \leqslant 8$ mg/mL). In a general population study, Jõgi et al. [26] found higher serum ECP levels in subjects with atopy or BHR, but no distinction was made between symptomatic and asymptomatic subjects. Gibson et al. [15] observed a higher number of eosinophils in sputum of children with BHR, although only if recent symptoms were also present.

In our population, we found similar results for blood eosinophils. However, interpretation of these similarities needs great cautiousness, since correlation between airway and blood eosinophilia is poor [27, 28]. In our study, we additionally observed more severe bronchial responsiveness in children with symptomatic BHR compared with those with asymptomatic BHR. Taken together, our observations suggest that eosinophilic inflammation particularly plays a role in more severe BHR that is more often accompanied by symptoms.

In the subgroup of atopic children, we observed higher levels of serum ECP in children with BHR both with and without the presence of wheeze. In contrast, blood eosinophils were only elevated if children had also been wheezing in the past 12 months. The interpretation of this observation is not fully clear. It may signify that activated blood eosinophils are a marker of atopy, whereas increased numbers of blood eosinophils *per se* more closely reflect ongoing airway inflammation with BHR and symptoms. Alternatively, the increased peripheral blood eosinophil counts and the possible enhanced eosinophil activation may account for the higher serum ECP. So far, only few studies have evaluated the relationship between serum ECP and asthma or BHR in a general population. Our results are consistent with clinical studies and suggest that BHR and asthma do not allow a description in a single inflammatory parameter [29]. This may be considered a confirmation of the complexity and heterogeneity of BHR and asthma.

A relevant source of bias in our study may have been the period reflected by either symptoms or blood eosinophilic markers. While symptoms dated a period of the past 12 months, eosinophilic markers likely reflect an inflammatory process that is represented over a much shorter period in time. This may well have underestimated the relationships between symptomatic BHR and blood eosinophilic markers.

Like others, we observed a relative high proportion of children with asymptomatic BHR (64%) [30–33]. In this study, we only considered a history of current wheeze as symptom indicative for asthma. This was confirmed by multiple logistic regression analyses, in which only this symptom was independently associated with the presence of BHR. Moreover, in children without wheeze, we observed twice as much dry cough in children without BHR (42%) compared with those with BHR (21%). Others have shown an overlap between wheeze and chronic cough, though wheeze is the strongest predictor for future wheeze or asthma [34, 35].

Corticosteroid treatment was neither an explanation for the high frequency of asymptomatic BHR, since none of these children used corticosteroids. We assume that underreporting of symptoms is the most likely explanation. This may particularly have occurred in children with less severe symptoms, or a larger time interval between symptoms. This is confirmed by our observation of a milder degree of BHR in asymptomatic BHR compared with symptomatic BHR. So far it is unclear if asymptomatic and symptomatic BHR should be considered separate conditions or reflect different grades of similar pathology. If the latter is the case, this may have implications for the development of symptoms in the future. A number of studies have shown an increased risk of developing asthma in subjects with asymptomatic BHR [36–38]. So far it is not clear which factors contribute to the development of symptoms. Nevertheless, subjects with asymptomatic BHR may be a relevant target group for prevention measures, though difficult to trace in the population.

In summary, children with BHR to HS were more frequently atopic to indoor allergens. If symptoms were present as well, they more often had multiple sensitizations, and higher levels of blood eosinophils and serum ECP compared with children without BHR and no symptoms. In atopic children, serum ECP was also higher in children with asymptomatic BHR. Furthermore, the degree of bronchial responsiveness was higher in symptomatic BHR compared with asymptomatic BHR. Taken together, our results suggest a relationship between eosinophilic inflammation, assessed by blood eosinophils and serum ECP and the degree of bronchial responsiveness to HS, which was present in atopic but not in non-atopic children.

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