

## Title Page

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iii. Abstract

**Background:** Airway hyperresponsiveness (AHR) is a common feature in asthma. The use of AHR in predicting active asthma or the persistence of AHR in childhood is poorly understood. By analyzing longitudinal connections including different measures of AHR, lung function, and inflammation markers, we sought to identify the best available method for predicting persistence of AHR and identification of later active asthma.

**Methods:** We tested 105 asthmatic children aged 3-7 years with fractional exhaled nitric oxide (FeNO), impulse oscillometry (IOS), and AHR evaluated by indirect methods (hypertonic saline and exercise challenge). Ten years later, 64 children participated in the follow-up visit and were tested with FeNO, IOS, spirometry, and methacholine challenge. At both study visits, blood samples were collected, and a questionnaire was completed.

**Results:** Asthma was in remission in 66% of patients at adolescence. AHR measured by hypertonic saline challenge at preschool age was associated with asthma symptoms (OR 10.2; 95%CI 2.8, 37.3) but not with AHR estimated with methacholine challenge 10 years later. AHR measured by exercise challenge was not associated with AHR or recent asthma symptoms in adolescence. Preschool eosinophilia continued until adolescence in 87% of patients but was not associated with AHR or subjective signs of asthma 10 years later. Wheezy preschoolers with atopy had a higher risk for AHR in adolescence (OR 4.1; 95%CI 1.0, 16.2).

**Conclusion:** Results from hypertonic saline challenge are associated with persistent asthma symptoms even after a decade. AHR measured by indirect methods at preschool age did not predict AHR in adolescence.

## Predictive value of childhood airway hyperresponsiveness to indirect stimuli: 10-year longitudinal study

### INTRODUCTION

Airway hyperresponsiveness (AHR) is a universally acknowledged feature of asthma<sup>1</sup> but has a controversial role as a predictive factor for asthma persistence<sup>2-4</sup>. Methods to measure AHR include the use of indirect (exercise, hypertonic saline, cold air) and direct (methacholine, histamine) stimuli.<sup>5</sup> In the direct mechanism, methacholine or histamine bind directly to the receptors on airway smooth muscle cells leading to muscle contraction. In contrast, indirect stimuli cause a release of mediators that provoke smooth muscle cells.

The method of measuring AHR might be of importance when analyzing the prognostic value of AHR. Increased AHR to methacholine in infancy is associated with persistent symptoms of asthma in later life.<sup>2</sup> In addition, we have previously shown that toddlers (age 6-24 months) that were reactive to methacholine more often had AHR to methacholine and to exercise challenge 5 years later than non-reactive toddlers.<sup>6</sup> There are thus far limited data on how AHR to indirect stimuli in early childhood predicts later AHR or asthma.

Although there are several known risk factors for asthma, their mutual long-term interactions are not fully understood. Eosinophilic inflammation has shown a causal connection to doctor-diagnosed asthma<sup>7</sup> but not to AHR.<sup>8</sup> However, such a connection between atopy and AHR in a pediatric population has been presented in an Australian cohort.<sup>9</sup> With regards to early wheezing, the connection appears to be controversial.<sup>6,10</sup> Although lung function measured with impulse oscillometry (IOS) in preschool children is a versatile prognostic tool, the association with later AHR remains unclear.<sup>11</sup>

We sought to estimate the prognostic value of measuring AHR with indirect stimuli (exercise and hypertonic saline) and the persistence of asthma symptoms from preschool age to adolescence. Furthermore, we sought to determine if AHR measured with direct stimuli in adolescence can be characterized by indirect measurements of AHR, lung function measured by IOS, blood eosinophilia, fractional exhaled nitric oxide (FeNO), or wheezing in early life.

## METHODS

Children aged 3 to 7 years (n=105) with newly diagnosed asthma confirmed by lung function measured by IOS were eligible for this prospective study of AHR in the Skin and Allergy Hospital of Helsinki. IOS was combined with an outdoor exercise challenge test and a bronchodilator test. The entry criteria included either increase of  $\geq 35\%$  in resistance at 5 Hz (R5) after exercise challenge or a decrease of  $\geq 35\%$  in R5 after bronchodilation test.<sup>11</sup> At this age, AHR was also estimated with a bronchial challenge test with hypertonic saline. Exclusion criteria included the use of systemic or inhaled corticosteroids in the previous 6 months, having seasonal asthma symptoms only, or a respiratory tract infection 2 weeks prior to lung function tests.

Altogether 64 (61%) children participated in the follow-up visit 10 years later; the children were aged 12 to 16 years at this time. Lung function was tested with spirometry and IOS and AHR was estimated with methacholine challenge. The protocol prohibited the use of corticosteroids or leukotriene receptor antagonists 2 months prior and bronchodilators 12 hours prior to the lung function measurements in adolescence. At both study visits, the families completed a detailed questionnaire. Wheezing, recurrent cough, rhonchi, or dyspnea during the previous 2 months was interpreted as active asthma in adolescence. Symptom-free individuals during a medication pause were considered to be in clinical remission.

This study complied with the principles of the Declaration of Helsinki and was approved by the Research Ethics Committee of the Helsinki University Central Hospital (139/13/03/2011). All parents provided written informed consent before enrollment and again at the follow-up visit in addition to the child's own assent.

### Procedures

Atopy was considered to be present if a child had a wheal diameter of  $\geq 3$  mm in skin prick tests. The tests covered sensitization to the 10 following local aeroallergens; birch, timothygrass, meadow fescue, mugwort, *Cladosporium herbarum*, dog, cat, horse, cow, and house dust mite. Peripheral blood eosinophil and neutrophil counts were obtained from standard complete blood counts. A blood eosinophil percentage of  $\geq 4\%$  was set as a limit for eosinophilia.

Oscillometry was performed at both study visits using the same apparatus (IOS Jaeger GmbH, Würzburg, Germany) and spirometry only at the 10-year follow-up visit using a pneumotachograph-based spirometer (Masterscreen Pneumo, Carefusion, Hoechberg, Germany). The baseline measurement of IOS at preschool age was followed by exercise challenge as described previously.<sup>12</sup> All measurements were combined with a bronchodilator test of inhalation of 300 µg salbutamol/albuterol (Ventoline, GSK, UK) (via Babyhaler) at preschool age and 400 µg (via Volumatic) in adolescence. ATS/ERS recommendations<sup>13</sup> for spirometry and principles of IOS<sup>14</sup> are described elsewhere.

The IOS parameters of interest at preschool age were respiratory resistance (R5) and respiratory reactance at 5 Hz (X5). In adolescence we recorded the following IOS and spirometry parameters: R5, X5, FEV1, and FEV1/FVC. The spirometry and preschool parameters were converted into age-, height-, and gender-adjusted z-scores based on Finnish reference values.<sup>15,16</sup> Limit for abnormal R5 and FEV1 is a z-score  $\geq 1.645$  and for X5 and FEV1/FVC is a z-score  $\leq -1.645$ .

Bronchial challenge with hypertonic saline was performed using IOS Jaeger GmbH equipment (Würzburg, Germany) following principles described earlier.<sup>17,18</sup> R5 was measured in triplicate at baseline and following a stepwise increasing dosage of 4.5% hypertonic saline aerosol via an ultrasonic nebulizer (Ultra-Air NE-U17; Omron, Matsusaka, Japan) for 0.5, 1, 2, 4, and 8 min, or until an increase of at least 35% was observed in R5 (PD<sub>35</sub>R5). The cumulative dose inhaled was determined by a weight method and PD<sub>35</sub>R5 by a dose-response curve. AHR was considered to be present if a significant increase in R5 was observed after any dose.

Paired measurements of FeNO were performed with a chemiluminescence analyzer (NIOX, Aerocrine AB, Solna, Sweden) following current recommendations<sup>19</sup> at both visits. After maximal inhalation, the child exhaled at a constant flow of 50 mL/s for  $\geq 6$  s, including a plateau phase of  $\geq 2$  s. Performance of the study subjects was enhanced by a balloon animation. The details of the procedure are described elsewhere.<sup>19</sup> The FeNO values were expressed as height- and gender-corrected z-scores.<sup>20</sup> A z-score  $> 2.0$  was considered abnormal.

The methacholine challenge test was performed using an automatic breath actuating dosimeter (Spira Elektro 2, Hämeenlinna, Finland) at the follow-up visit. By using the dose-response curve, the provocative dose of inhaled methacholine producing a 20% fall in FEV1 (PD<sub>20</sub>FEV1) was determined.<sup>1</sup> The procedure included five cumulative dose steps. The test was considered positive (AHR) if the PD<sub>20</sub>FEV1 was <400 µg.

SPSS 25 (SPSS, Inc, Chicago, USA) was used for data analysis. Continuous variables are reported as means and standard deviations and dichotomous variables as percentages. Spearman's correlation test was used for linear correlations, Chi-square and Kruskal-Wallis tests for categorical variables, paired sample *t*-tests for paired variables, and Mann-Whitney for independent samples. FeNO and methacholine were non-normally distributed, hence non-parametric analysis with Kendall-Tau were applied. Multivariate analysis was performed using linear regression. A *P*-value ≤0.05 was considered significant. Results were further verified by the biostatistics department of the university.

## RESULTS

A decrease in the occurrence of wheezing, number of blood eosinophils, FeNO levels, and baseline R5 were detected during followup (Table 1), while atopy and baseline X5 levels increased. Parental asthma was smoking were significantly more frequent among the preschool dropouts than in the preschool participants ( $P=0.017$  and  $P=0.026$  respectively). There were otherwise no significant differences between the participants and the dropouts. Asthma was in clinical remission in 66% of the children at adolescence. The most common asthma symptom among the adolescents was recurrent cough and the symptoms most often appeared during respiratory infection (Table 2).

AHR measured by hypertonic saline or by exercise challenge at preschool age did not predict the outcome of methacholine challenge 10 years later (Figure 1b) and no association between these three was observed. Study subjects sensitized to furry animals or who had both wheezing and atopy at preschool age had an increased risk for AHR in adolescence (Table 3). It should be noted, however, that wheezy children were more often sensitized to furry animals ( $P=0.030$ ) than non-wheezers. Abnormal baseline X5 (z-score  $\leq -1.645$ ) at preschool age was associated with later AHR (OR 6.9; CI 1.1, 42.1). Figure 2 presents the associations between preschool R5 and X5 to AHR severity in adolescence. All preschoolers with abnormal baseline X5 had AHR in hypertonic saline challenge at preschool age and 67% also had AHR during methacholine challenge in adolescence.

Blood eosinophilia persevered in 87% of the children 10 years later. Our data showed a negative correlation between preschool blood eosinophil levels and adolescent methacholine results ( $R=-0.227$ ;  $P=0.022$ ). Although blood eosinophilia at preschool age did not predict asthma or AHR, it predicted a positive FeNO result in adolescence (Figure 1a). Wheezing in the past year at preschool age was also a predictive factor for positive FeNO (OR 5.7; 95% CI 1.1, 28.9). Wheezy children with atopy had a further increased risk for positive FeNO in adolescence (OR 11.3; 95% CI 3.2, 40.0). Positive FeNO at preschool age was not associated with any other factors assessed at any age (Figure 2b).

Recent asthma symptoms in adolescence were reported by 33% patients with a positive methacholine challenge and by 26% of those with a negative result. AHR to hypertonic saline at preschool age was associated with asthma symptoms (OR 10.2; 95% CI 2.8, 37.3) in adolescence (Figure 1b). However, AHR measured with exercise challenge at preschool age

was not significantly associated with asthma symptoms 10 years later. A significant association was observed between asthma symptoms in later life and both abnormal baseline X5 (OR 12.1; 95% CI 1.3, 111.1) and R5 (z-score $\geq$ 1.645) (OR 4.4; 95% CI 1.1, 17.4) at preschool age. Sensitization to furry animals was not significantly associated with asthma symptoms 10 years later. However, an association was observed between asthma symptoms reported from animal contact in adolescence and dog (OR 6.5; 95% CI 1.8, 23.4) or cat (OR 7.6; 95% CI 2.2, 26.1) sensitization in early childhood. Preschool blood eosinophilia, positive FeNO result, and wheezing alone or combined with atopy did not have any predictive value of subjective measures of asthma in later life.

## DISCUSSION

We observed that although preschool AHR measured by hypertonic saline challenge does not identify adolescents with AHR, this challenge successfully finds those who continue to experience asthma symptoms in adolescence. AHR measured by exercise challenge did not predict the perseverance of either AHR or asthma symptoms, which proves that methods for detecting AHR are important when assessing the prognostic value of AHR. Furthermore, our analyses demonstrate that atopy together with wheezing at preschool age is associated with AHR in later life. Blood eosinophilia was not an effective predictor of AHR or active asthma.

According to the ERS task force, the primary indications for using hypertonic aerosols are to detect AHR associated with active asthma or exercise-induced asthma and to evaluate the patient's responsiveness to treatment with anti-inflammatory drugs.<sup>21</sup> Our study took this a step further and demonstrated that AHR measured with hypertonic saline at preschool age was associated with active asthma even a decade later. Also, the connection with preschool abnormal baseline X5 supports the evidence that early-onset AHR is linked to poorer prognosis and dysfunction of small airways.<sup>22</sup> One specific shortcoming to the test was the difficulty in execution. Compared to methacholine challenge, where methacholine is delivered promptly to airways, during hypertonic saline challenge children must endure an unpleasant airway irritant for minutes.<sup>23</sup> Many of the children that failed to perform the challenge also dropped out of the entire study. A re-evaluation of the test protocol and deliberated improvements may address these problems. In contrast to hypertonic saline challenge, our study revealed a poor correlation between methacholine challenge results and recent asthma symptoms. Hence, using methacholine challenge following asthma exacerbations or to assess the effectiveness of asthma therapy might not be an ideal practice.

No correlation or association was found between exercise challenge combined with IOS at preschool age and methacholine challenge results in adolescence. Use of exercise challenge as one of the inclusion criteria might have affected the results and a control group would have made the setting more reliable. Instead, IOS alone showed good potential as a screening method for persistent asthma with connection to both objective and subjective signs. These prognostic features of baseline R5 and X5 (both representatives of small airways) were further elucidated when compared to our previous study, when these features were evaluated with a control group.<sup>11</sup> However, the predictive value of IOS parameters and hypertonic saline are more indicative than conclusive and further studies are necessary.

In our study population, blood eosinophilia persevered or at least reappeared after 10 years. Nevertheless, eosinophilia at preschool age was neither associated with abnormal lung function nor with clinical manifestations of asthma in adolescence. Sixty-six percent of our study patients at adolescence were in remission, which is comparable the values (60-64%) presented in the literature.<sup>24,25</sup> The children in our study had mainly moderate asthma; this can affect the findings as severe asthmatics are rarely in remission.<sup>25</sup> Although eosinophilic inflammation is associated with later asthma, early blood eosinophilia may not be the best predictive factor for mild-to-moderate asthma.<sup>26</sup> Furthermore, on the basis of our results, FeNO at preschool age had no prospective association with any other measure of asthma. When compared to inexpensive and more informative blood eosinophil count, value of early FeNO measurement seems dubious.<sup>27</sup>

Our results show that different asthma phenotypes can be recognized in early childhood. Individuals with early AHR measured by hypertonic saline continue to experience symptoms and to have defects in lung function in adolescence. These individuals may require more consistent followup. In contrast, children with atopy and blood eosinophilia were symptom-free in adolescence.<sup>28</sup> A longer follow-up or a cohort including severe asthmatics may reveal a different outcome, particularly regarding the recurrence of symptoms later in adulthood for atopic female subjects.<sup>5,29</sup> Physicians should also consider that applying only one type of objective test may not reveal all asthmatics and that occasionally applying a larger set of tests is required to identify all asthma phenotypes. Nonetheless, this study and others before have shown that asthma originates early in life<sup>29-31</sup> and objective measures cannot be delayed to schoolage.

We acknowledge that the findings of the present study are limited by the relatively small number of study subjects. However, the results are supported by the fact that data were collected prospectively, all pulmonary testing was conducted in the same center by the same team, and all results were evaluated by the same clinical physiologist. Further, unlike previous studies, our data allowed comparison of four different objective measures of pulmonary function, which offers a unique opportunity to characterize different asthma phenotypes.<sup>22,24</sup>

In conclusion, our data indicates that AHR measured by indirect methods (hypertonic saline and exercise challenge) at preschool age is not connected to AHR measured by a direct method (methacholine) in adolescence. We also observed that AHR measured with hypertonic saline challenge is a viable tool to identify adolescents with active asthma 10 years earlier. Active asthma in adolescence does not appear to be determined by early blood eosinophilia.

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Table 1. Demographics.

|                                                  | Preschool    |                         | Adolescence   |
|--------------------------------------------------|--------------|-------------------------|---------------|
|                                                  | Participants | Drop-outs               |               |
| n                                                | 64           | 41                      | 64            |
| Female, n (%)                                    | 21 (33)      | 12 (29)                 | 21 (33)       |
| Age, years [range]                               | 5.62 [3;7]   | 5.55 [3;7]              | 14.22 [12;16] |
| Wheeze during previous year, n <sup>a</sup> (%)  | 53 (83)      | 39 (95)                 | 7 (11)        |
| Parental asthma, n <sup>†</sup> (%)              | 15 (24)      | 19 (46)                 | 18 (28)       |
| Parental smoking, n <sup>†</sup> (%)             | 21 (33)      | 23 (56)                 | 16 (25)       |
| Positive SPT, n (%)                              | 45 (70)      | 35 (88)                 | 59 (92)       |
| Blood eosinophils, %, (SD)                       | 6.9 (4.8)    | 8.0 (4.6)               | 5.2 (4.2)     |
| Blood eosinophils $\geq 4\%$ , n (%)             | 53 (83)      | 34 (87)                 | 34 (54)       |
| FeNO z-score, (SD)                               | 2.5 (1.6)    | 2.4 (1.6)               | 2.1 (2.6)     |
| FeNO, z-score $\geq 2.0$ SD, n (%)               | 36 (60)      | 25 (63)                 | 31 (49)       |
| FEV1 z-score, (SD)                               |              |                         | -0.5 (1.2)    |
| FEV1, z-score $\leq -1.645$ SD, n (%)            |              |                         | 9 (14)        |
| R5, kPa/L/s, (SD)                                | 0.89 (0.22)  | 0.72 (1.30)             | 0.36 (0.09)   |
| X5, kPa/L/s, (SD)                                | -0.29 (0.17) | -0.30 (0.13)            | -0.09 (0.03)  |
| Positive exercise challenge <sup>‡</sup> , n (%) | 49 (77)      | 23 (70)                 |               |
| PD <sub>35</sub> R5 $\leq 23$ g, n (%)           | 27 (42)      | 12/30 (40) <sup>§</sup> |               |
| PD <sub>20</sub> FEV1 $\leq 400$ $\mu$ g, n (%)  |              |                         | 17 (27)       |
| Active asthma <sup>¶</sup> , n (%)               |              |                         | 22 (34)       |
| Asthma in remission <sup>++</sup> , n (%)        |              |                         | 42 (66)       |

<sup>†</sup>Self-reported, <sup>‡</sup>exercise challenge was interpreted positively with the increase of  $\geq 35\%$  in R5<sup>§</sup> 11 preschool dropouts refused to perform hypertonic saline challenge, <sup>¶</sup>Asthma defined as asthma symptoms during previous 2 months, <sup>++</sup>remission defined as lack of symptoms during a 2 months medication pause.

Abbreviations: FeNO, Fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 s; ISO-BMI, sex- and age-adjusted body mass index for children; PD<sub>20</sub>FEV1, 20% decrease in the forced expiratory volume in 1 second in methacholine challenge; PD<sub>35</sub>R5, decrease in the respiratory resistance at 5 Hz in hypertonic saline challenge; R5, respiratory resistance at 5 Hz; SPT, skin prick test; X5, reactance at 5 Hz.

Table 2. Self-reported asthma symptoms and the use of asthma medication during last 2 months in adolescence. n=64

|                                | n (%)      |
|--------------------------------|------------|
| Symptoms                       | 22 (34)    |
| Recurrent cough                | 7/22 (32)  |
| Wheeze                         | 5/22 (23)  |
| Rhonchi                        | 3/22 (14)  |
| Dyspnea                        | 7/22 (32)  |
| Symptoms during the following: |            |
| Respiratory infection          | 18/22 (82) |
| Exertion                       | 11/22 (50) |
| Pollen season                  | 11/22 (50) |
| Animal contact                 | 7/22 (32)  |
| Medication                     | 23 (36)    |
| Inhaled corticosteroids        | 15/23 (65) |
| Beta-agonists                  | 22/23 (96) |
| Combination                    | 6/23 (26)  |
| Leukotriene antagonists        | 6/23 (26)  |

Table 3. Predictive value of preschool skin prick test results for airway hyperresponsiveness in adolescence. Abbreviations: OR, odds ratio; PPV, positive predictive value.

| Predictive factor         | OR (95%CI)       | PPV | P-value |
|---------------------------|------------------|-----|---------|
| Atopy, n=45               | 4.3 (1.0, 20.8)  | 88% | 0.049   |
| Wheeze and atopy, n=39    | 4.1 (1.0, 16.2)  | 82% | 0.031   |
| Sensitized to dog, n=29   | 10.0 (2.5, 40.0) | 82% | <0.001  |
| Sensitized to cat, n=24   | 4.8 (1.5, 15.6)  | 65% | 0.007   |
| Sensitized to horse, n=22 | 3.1 (0.9, 11.2)  | 46% | 0.078   |

x. Figure legends

Figure 1 a) Conventional markers. b) Lung function markers. N=64. Arrows represent prospective associations between preschool and adolescence(10-year follow-up time). Emphasis is on continuous variables, but dichotomous used when necessary. Only the z-scores of FEV1, FEV1/FVC, R5, and X5 were included from spirometry and impulse oscillometry indices. Atopy was defined as positive skin prick tests. Wheezing at preschool age in the last year as reported by parents. Self-reported asthma symptoms and use of asthma medication in adolescence from previous 2 months. FeNO; fractional exhaled nitric oxide. Confounding effect of age, gender, and environmental tobacco smoke was excluded using general linear model analysis.

Figure 2. Distribution of preschool baseline a) R5 and b) X5 values among the severity groups of methacholine challenge in adolescence. Bonferroni corrected *P*-values. Severe reaction <150 µg, moderate reaction 150-600 µg, mild reaction 601-2600 µg. Abbreviations: AHR, Airway hyperresponsiveness; R5, resistance at 5 Hz; X5, reactance at 5 Hz.



