

ORIGINAL ARTICLE

Elevated inflammatory markers at preschool age precede persistent wheezing at school age

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Abstract

Background: Wheeze is a heterogeneous symptom in preschool children. At preschool age it is hard to predict whether symptoms will pass or persist and develop into asthma. Our objective is to prospectively study whether inflammatory markers in exhaled breath condensate (EBC) and pre- and post-bronchodilator interrupter resistance (Rint) assessed at preschool age, are associated with wheezing phenotypes at school age.

Methods: Children (N = 230) were recruited from the Asthma DEtection and Monitoring (ADEM) study. At preschool age [mean (SE): 3.3 (0.1) yr], pre- and post-bronchodilator Rint was assessed. EBC was collected using a closed glass condenser. Inflammatory markers (IL-2, IL-4, IL-8, IL-10, sICAM) were measured using multiplex immunoassay. Wheezing phenotypes at 5 yr of age were determined based on longitudinal assessment. Children were classified as: never (N = 47), early-transient (N = 89) or persistent wheezers (N = 94).

Results: Persistent wheezers had elevated levels of all interleukins at preschool age compared to children who never wheezed ($p < 0.05$). EBC markers did not differ between the persistent and transient wheezers. There was no marked difference in Rint between wheezing phenotypes.

Conclusions: We demonstrated that 5 yr old children with persistent wheeze already had elevated exhaled inflammatory markers at preschool age compared to never wheezers, indicating augmented airway inflammation in these children.

In preschool children, wheeze is a common respiratory symptom, though with a complex and heterogeneous character. Whereas the majority of wheezing preschool children will experience transient symptoms, around 30% of the children have persistent wheeze that develops into asthma (1). Currently it is not possible to easily distinguish children with transient symptoms from persistent wheezers. This leaves physicians with a challenging task concerning the diagnosis and management of preschool wheeze. Different wheezing phenotypes have been defined that classify children on basis of time course, frequency, severity, or triggers of their symptoms (2, 3). A widely used classification system is developed by researchers of the Tucson birth cohort study, dividing children into early transient, late onset, persistent and non-

wheezing phenotypes (1). Although it is generally assumed that these phenotypes have a different underlying pathophysiology, research in this direction is hampered due to problems with measurements of airway inflammation and lung function in young children. Most lung function measurements are hard to assess in young children since active cooperation is necessary. Further, the currently available techniques to measure airway inflammation such as bronchial biopsies and bronchoalveolar lavages are invasive and therefore not appropriate for routine use. To avoid these invasive techniques, there is an increasing interest in the non-invasive assessment of inflammatory markers in exhaled breath. Besides measurements of exhaled nitric oxide (4), the analysis of solutes in exhaled breath condensate (EBC) gained

popularity. We previously showed that the analysis of markers in EBC is a non-invasive and feasible technique to assess airway inflammation in wheezing preschool children (5, 6). Different forms of airway inflammation are believed to play a significant role in preschool wheeze. There is increasing evidence that in children with transient, virus-induced wheeze, neutrophil mediated inflammation plays a central role (7–9). In asthmatic children, a T helper (Th) 2 mediated immune response is believed to be of importance (10). Therefore, it can be hypothesised that EBC levels of interleukin (IL)-8, the major neutrophil chemo attractant, are elevated in children with transient wheeze, while EBC levels of IL-4, a prototype Th2 cytokine, are elevated in children with persistent symptoms, prone to develop asthma. In case children with distinct wheezing phenotypes differ in these and/or other exhaled inflammatory markers at preschool age, this technique might be useful to early identify children at risk to develop asthma. However, this has not been studied yet.

Besides measurements of airway inflammation, lung function measurements can give insight in the underlying pathophysiology of preschool wheeze. A potential useful lung function technique that can easily be applied in young children is the measurement of airway resistance using the interrupter resistance technique (Rint) (11–13). Previous studies demonstrated increased airway resistance (assessed with Rint and forced oscillation technique) in children with persistent wheezing symptoms compared to children with transient wheeze (12, 14). In these studies, wheezing patterns and airway resistance measurements were both assessed at school age. The potential role of preschool Rint to predict the future course of wheezing symptoms is hardly studied.

The aim of this study was to define prospectively whether inflammatory markers in EBC (including IL-4 and IL-8) and Rint values assessed at preschool age, are associated with wheezing phenotypes at the age of 5 yr.

Methods

Study design

Children were included from the ADEM study (Asthma DEtection and Monitoring study). This case control study started in 2006 at the Maastricht University Medical Centre, the Netherlands. In this study, children who experienced at least two wheezing episodes during life [$N = 202$, assessed by the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (15)], and control subjects ($N = 50$) who never wheezed were included at 2 and 3 yr of age and were prospectively studied until 6 yr of age. The current analysis is based on data from inclusion until 5 yr of age. The primary objective of the ADEM study is to develop a non-invasive instrument for an early asthma diagnosis by using exhaled inflammatory biomarkers and early lung function measurements. The study protocol was described in detail before (16). Ethical approval was obtained from the Dutch National Medical Ethical Committee (CCMO: NL17407.000.07/2007-001817-40, The Hague, The Netherlands). All parents gave written informed con-

sent. This study is registered by clinicaltrial.gov (NCT 00422747).

Measurements

At inclusion (age 2 and 3 yr), measurements of lung function and exhaled inflammatory markers were performed. If applicable, inhaled corticosteroids (ICS) administration was stopped 4 weeks before the start of the measurements. In case of symptoms of an airway infection, a measurement was postponed for 4 weeks. Physical exercise and solid foods were not allowed 1 h before the measurements.

Atopy

Venous blood was collected and serum was tested for specific IgE against a mixture of inhalant and food allergens including hen's egg, cow's milk, peanut, shrimp, house dust mite, cat, dog, birch and a mix of grasses (Phadiatop infant[®], Phadia, Uppsala, Sweden). A child was considered atopic when the value of the Phadiatop Infant test equals or exceeded 0.35 kU/l.

Lung function measurements

To assess lung function we measured interrupter resistance, by using the MicroRint device (Micro Medical Ltd, Rochester, UK). Children were sitting in an upright position and were asked to breathe tidally through a facemask. Interruptions were made on the expiratory peak flow and the median value of at least five successful airflow interruptions was used for analysis. To assess the reversibility to a short acting beta-2 agonist, Rint measurements were repeated 15 min after inhalation of 300 µg of salbutamol inhaled via an Aerochamber[®] (Airomir[®], Teva Pharma NL, Haarlem, the Netherlands). Reversibility was expressed as absolute difference in interrupter resistance before and after the application of salbutamol ($R_{\text{int, before}} - R_{\text{int, after}}$).

Exhaled breath condensate

A closed glass condenser system with a breath recirculation, developed at our institute, was used to collect EBC (16). This efficient condenser system ensures a sufficient volume of EBC collected in a short collection-time (10 min). The sampling- and collection procedures were described previously (16). Inflammatory markers in EBC [IL-2, IL-4, IL-8, IL-10, soluble Intercellular Adhesion Molecule 1 (sICAM)] were assayed with the multiplex immunoassay (Luminex Corporation, Austin, TX, USA) (17). Samples with levels of EBC markers below the detection limit received a randomly generated value taken from the uniform distribution between zero and the detection limit (6).

Wheezing phenotypes

Data on symptoms of wheeze were collected yearly from inclusion until 5 yr of age by using the ISAAC questionnaire (15). Data from birth until inclusion were reclaimed retrospectively. Based on these longitudinal data, children were divided into three wheezing phenotypes: 'never wheeze' (children who never wheezed during the first 5 yr of life); 'early transient wheeze'

(at least two wheezing episodes during the first 3 yr of life, no wheeze in the 4th and 5th year) and 'persistent wheeze' (multiple wheezing episodes during the first 5 yr).

Statistical analysis

Data were analysed with SPSS 16.1 (SPSS inc., Chicago IL, USA). Differences in baseline characteristics, EBC markers and Rint values among wheezing phenotypes were evaluated using the Kruskal Wallis (overall test), Mann–Whitney or Chi-square analysis. Adjustment for multiple testing was conducted via the false discovery rate (FDR). A significance level of 5% was used.

Power analysis

In an earlier study in children with asthma and healthy controls aged 6–16 yr, we found differences in concentrations of IL-2, IL-4, and IL-8 ranging from 2.9 to 13.2 pg/ml (see reference for accompanied standard deviations) (18). If we assume comparable differences in the present population, we need at least 43 children in each category of wheeze to detect a difference in mean concentration of 2.9 pg/ml, with a power of 90% and a two-sided alpha of 0.05.

Results

Baseline characteristics and wheezing phenotypes

Of the 252 children included in the ADEM study, longitudinal data of respiratory symptoms were available in 230 children (91%). Reasons for lack of longitudinal data were: lost-to follow up due to personal constraints (N = 19), and not yet reached the age of 5 yr at the time of analysis (N = 3). Children who were excluded due to lack of data did not differ in Rint, EBC markers and baseline characteristics compared to included children, except for age. The included children were divided into those who never wheezed (N = 47, 20%), those with early transient wheeze (N = 89, 39%) and those with persistent wheeze (N = 94, 41%). The sampling procedure of Rint and EBC was successfully employed in 97% (N = 223) and 98% (N = 226) of the children, respectively. Baseline characteristics are given in Table 1. More than half of the children with persistent wheeze used short acting beta-2 agonist as rescue medication, whereas ICS was used in 27% of the children, respectively. As can be expected, these percentages were significantly higher compared to the never and transient wheeze group. Seven of the persistent wheezers were not able to stop ICS prior to the measurements. A sensitivity analysis excluding these children did not reveal different results. Therefore these children were kept in the analysis. Moreover, two children of the never wheeze group used inhalation medication for recurrent cough (one child used ICS and a short acting beta-2 agonist, the other one only used a short acting beta-2 agonist as rescue medication). As these children might be misclassified, we performed an additional analysis excluding these children. Since no differences in results were found, these children were kept in the analysis. Eczema was more present in

Table 1. Baseline characteristics of wheezing phenotypes

	Never Wheeze	Early Transient Wheeze	Persistent Wheeze
Study population, N	47	89	94
Median age (Inter Quartile Range), in years†	3.5 (2.8–3.8)	3.4 (2.9–3.8)	3.1 (2.8–3.7)
Gender: male/female, in N	25/22	49/40	53/41
Atopy, in %‡	29	23	27
Eczema, in %	19	33	42*
ICS use, in %	2	10	27**
Short acting beta-2 agonist use, %	4	21*	54**

ICS, inhaled corticosteroids. *False discovery rate (FDR) corrected p-value < 0.05 relative to never wheezers, **FDR corrected p-value < 0.05 relative to never wheezers and transient wheezers. †Age at time of inclusion (time point of assessing inflammatory markers and Rint), ‡Atopy is defined as a positive Phadiatop Infant test.

persistent wheezers compared to never wheezers. There were no differences in age, gender and atopy between wheezing phenotypes.

Exhaled biomarkers

Interleukins and soluble adhesion molecules could be detected in respectively 96% and 97% of the samples. Apart from sICAM, all interleukins (IL-2, IL-4, IL-8 and IL-10) were significantly different in the wheezing phenotypes (corrected for multiple testing). Figure 1a–b illustrates that the overall difference in IL-2, IL-4, IL-8 and IL-10 was mainly due to higher levels in the persistent wheeze group compared to the never wheeze group (FDR corrected p-values: 0.01, <0.01, 0.02, 0.01, respectively). Children with transient wheeze had insignificantly higher levels of all interleukins (IL-2, IL-4, IL-8 and IL-10) compared to children who never wheezed (FDR corrected p-values: 0.06, 0.07, 0.08, 0.06, respectively, Fig. 1a–b). No differences in markers were found between the children with persistent wheeze compared to children with transient symptoms (FDR corrected p-values > 0.05, Fig. 1a–b). In a sub-analysis, children with atopy and/or eczema were compared to children without atopy and/or eczema. There were no differences in biomarkers between atopic children and children without atopy, neither in the total group nor when stratified for wheezing phenotypes. Comparable results were found for eczema. Moreover, there were no differences in biomarkers between males and females, neither in the total group nor when stratified for wheezing phenotypes.

Interrupter resistance

The overall test showed a borderline significance of differences in baseline Rint values between wheezing phenotypes (FDR corrected p-value = 0.05). This overall mild difference

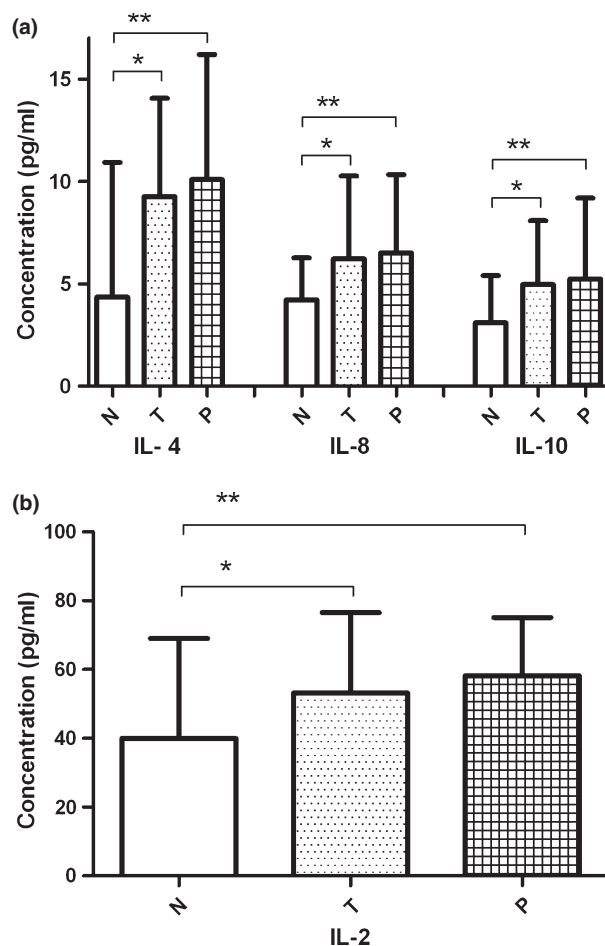


Figure 1 (a–b) Markers in EBC in never wheeze (N, open bar), transient wheeze (T, spotted bar) and persistent wheeze groups (P, chequered bar). Pair-wise comparisons; ** p-value < 0.05, *p-value < 0.10 (False discovery rate corrected).

was due to higher levels of Rint in children with persistent wheeze compared to never wheezers and children with transient symptoms (FDR corrected p-values: 0.06, 0.07, Fig. 2). There were no differences in reversibility between the wheezing phenotypes, as noted by a median change (Inter Quartile Range) of Rint after a bronchodilator in never wheezers; 0.1 (0.0–0.4) kPa·s/l, in transient wheezers; 0.2 (0.1–0.4) kPa·s/l, and in persistent wheezers; 0.3 (0.1–0.5) kPa·s/l, $p = 0.15$. A sub-analysis revealed no differences in both lung function parameters between children with atopy compared to children without atopy, which holds true for eczema as well.

Discussion

We demonstrated that inflammatory markers in EBC at preschool age were related to wheezing phenotypes at 5 yr of age: children with a persistent wheezing phenotype already had elevated levels of most cytokines/chemokines in EBC at preschool age compared to the never wheezing phenotype. However, based on EBC measurements at 2 and 3 yr, it was

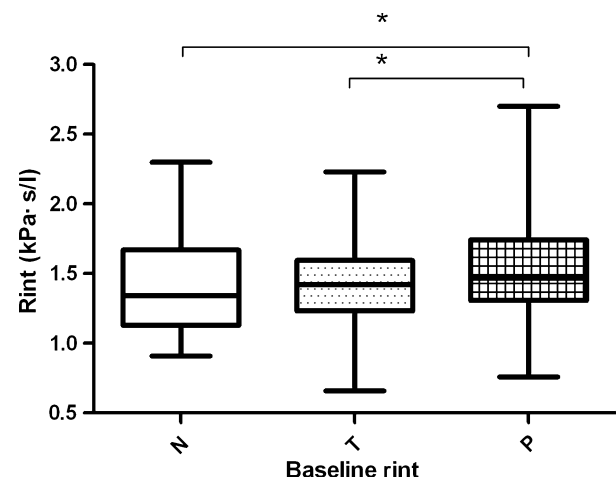


Figure 2 Baseline Rint values in never wheeze (N, open bar), transient wheeze (T, spotted bar) and persistent wheeze groups (P, chequered bar). Pair-wise comparison; *p-value < 0.10 (False discovery rate corrected).

difficult to differentiate between the transient and persistent wheezing phenotype. No marked difference in Rint was found between the wheezing phenotypes.

To our knowledge, this is the first study that prospectively explored whether non-invasive inflammatory markers in EBC are related to wheezing patterns in young children. We found that children with different wheezing patterns have elevated levels of multiple inflammatory markers at preschool age compared to children who never wheezed, with the highest levels in children with persistent wheeze. This is in accordance with a previous cross-sectional analysis of our data, in which we found elevated levels of multiple inflammatory markers in wheezing preschool children compared to healthy controls (6). The elevated inflammatory markers may point to a broad inflammatory response in children with persistent wheeze, with involvement of Th1/2 cytokines and the chemokine IL-8. This multiple inflammatory response is in agreement with the hypothesis that in wheezing preschool children multiple immune mechanisms are involved that lead to a sequence of inflammatory responses (19). Although levels of markers were elevated in wheezing children compared to children who never wheezed, these markers were not able to differentiate children with transient symptoms from children with persistent symptoms prone to develop asthma. As transient wheezers are believed to have virus-induced symptoms in which neutrophilic airway inflammation plays a central role (7–9), we would have expected elevated levels of IL-8, a neutrophil chemo attractant, in the group of children with transient wheeze. However, this was not the case. A possible explanation is that we did not measure EBC markers during an acute episode of wheeze. It is conceivable that a more prominent difference in levels of IL-8 will be found between phenotypes when EBC is measured during an acute, virus-induced episode of wheeze.

In our study baseline Rint values assessed at preschool age, were slightly elevated in children with persistent wheeze

compared to children who never wheezed and children with transient symptoms. However, there was a considerable overlap in Rint distributions between the groups. Neither did we find a clear difference in reversibility, expressed as absolute difference in Rint before and after salbutamol, between wheezing phenotypes. We have studied the reversibility on a continuous scale and did not use a cut-off point since such an analysis would be less sensitive and would depend on the cut-off point chosen. The ATS/ERS statement noted that a clinical relevant cut-off point for reversibility still needs to be established (20). In previous performed studies a more pronounced difference in airway resistance (assessed with Rint and forced oscillation technique) between wheezing phenotypes was found, with elevated levels in children with persistent wheeze compared to children with transient symptoms (12, 14). This might indicate that children with persistent symptoms experience airway obstruction, a characteristic of asthma, while children with transient, virus-induced symptoms do not have a diminished airway calibre at school age. In contrast to our study these studies assessed Rint values and wheezing phenotypes at the same age. They did not prospectively study whether Rint assessed at preschool age was related to the future course of wheezing symptoms. Recent data from the 'Prevention and Incidence of Asthma and Mite Allergy (PIAMA)' birth cohort study demonstrated that Rint assessed in 4-yr-old children was associated with wheezing at age 6. However, at the age of 8 yr this association vanished (21). Taken together, we might conclude that although airway resistance is increased in school children with current, persistent symptoms compared to children who experienced symptoms in the past, the power of preschool Rint to predict whether wheezing symptoms will persist or fade out is limited.

Exhaled breath condensate markers and Rint levels did not differ between a transient and a persistent wheezing phenotype. The lack of this discriminative ability can be due to several reasons. At first, it might be explained by methodological issues. One may argue that selection bias has occurred due to incomplete data as a result of 'lost-to-follow-up' or unsuccessful measurements. However, this is very unlikely since children who did not complete the study did not differ in EBC markers at preschool age compared to children who completed the study. Besides, the success rate of EBC collection was high and general reasons for failure (fear and unwillingness of the child) were unrelated to EBC levels. A second methodological issue might be the fact that assessing markers in EBC can still be optimised. Although an American/European Taskforce has presented recommendations about the methodology of EBC collection (22), there is still considerable room for improvement in collection methods and assays. Our research group has substantial experience in measuring exhaled inflammatory markers in children and progress has been made (5, 6, 18, 23). This has resulted in the development of an efficient glass condenser system that enables the collection of EBC in a short collection time in a reproducible way (5). Moreover, we can accurately measure different cytokines in a small sample of EBC (60 µl) by using highly sensitive multiplexed liquid bead arrays (17). This

makes our technique highly suitable for young children. However, the validity of measuring EBC markers may be further increased by controlling for factors such as expiratory flow rate or dilution factor. Although we did not consider these factors 4 yr ago when the first EBC samples were collected for the ADEM study, our research group is currently studying the influence of these factors. A third methodological issue might be possible misclassification of wheezing phenotypes, since phenotypes were based on parental-reported symptoms. However, recall bias was minimised by annual assessment of symptoms, and the ISAAC questionnaire that was used is validated and commonly used for similar purposes (15).

Besides methodological issues, there is an alternative and perhaps more plausible explanation for the lack of differences in exhaled inflammatory markers in distinct wheezing phenotypes. Preschool wheeze is a heterogeneous condition and there are multiple ways to classify wheezing children, as reviewed by Spycher et al. (3). In this review, they questioned whether wheezing phenotypes based on a single characteristic, such as retrospective symptom history, represent distinct underlying disease entities. Our data might support this issue. We demonstrated that children with wheezing patterns classified based on course of symptoms, do not markedly differ in underlying airway inflammation assessed by exhaled inflammatory markers at preschool age. Instead of classification on symptom history, it would be interesting to study whether wheezing preschool children could be divided into 'inflammatory' phenotypes, and whether these phenotypes differ in response to treatment and prognosis in later childhood. If that is the case, classifying children based on their inflammatory 'breath-print', might better separate the underlying diseases causing wheezing symptoms in children. This can improve diagnosis and treatment of preschool wheeze. Moreover, these phenotypes can already be assessed at preschool age whilst classifying children on basis of wheezing patterns can only be determined retrospectively.

In conclusion, we demonstrated that children with persistent wheeze at 5 yr of age already have elevated levels of exhaled inflammatory markers compared to children without wheeze at preschool age. This finding points to an increased inflammatory response in these preschool wheezers. Classifying children on basis of inflammatory- and lung function characteristics instead of retrospective symptom history, might provide a better biological basis to unravel the heterogeneous character of wheeze. We demonstrated that there are potential, non-invasive and feasible techniques available that can provide information on underlying airway inflammation and airway obstruction in preschool children. Whether the use of these techniques to classify wheezing preschool children is helpful to improve diagnosis and treatment of preschool wheeze needs to be proven in future research.

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Competing interests

The authors declare that they have no competing interests.

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