

Original article

Neutrophilic airway inflammation is a main feature of induced sputum in nonatopic asthmatic children

Background: Asthma phenotypes are well described among children. However, there are few studies comparing airway inflammation in different clinical presentations of pediatric asthma. We tested the hypothesis that nonatopic asthma is associated with a predominant noneosinophilic inflammation in the airways, as assessed by induced sputum. The objective of this study was to evaluate the cytological characteristics of induced sputum (IS) in atopic (AA), nonatopic asthmatics (NAA) and nonatopic nonasthmatic children (NANA). **Methods:** Of 90 selected children, 77 met eligibility criteria for performing IS and were classified as: AA, $n = 28$, NAA, $n = 29$ and NANA, $n = 19$. Subjects answered to a set of ISAAC-based questions and were skin-tested for common aeroallergens. A defined series of exclusion criteria was applied.

Results: Induced sputum was obtained from 54 (70.1%) subjects (21 AA, 20 NAA and 13 NANA). Demographic data and mean FEV₁ were similar in the three groups. The proportion of eosinophils [median, inter quartile range (IQR)] was significantly higher in the sputum of AA [(6.0).12)] compared with NAAs [0 (2)] and NANAs [0 (1)], $P < 0.001$. The proportion of children with sputum eosinophilia (eos $> 3\%$) was also significantly higher in AA (71.4%) when compared with NAA (28.6%); none of the NANA had sputum eosinophilia. Nonatopic asthmatic children had significantly higher proportions and absolute number of neutrophils than AA and controls.

Conclusions: The results suggest that nonatopic children present IS with a cell pattern that is predominantly neutrophilic while eosinophilia is the hallmark of airway inflammation in the majority of atopic wheezing children not treated with inhaled steroids.

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Asthma is a disease explained by multiple variables, affected by a combination of genetics and the environment, with all its socioeconomic and cultural influences. Current guidelines define asthma as a condition characterized by variable airflow limitation, airway hyper-responsiveness and airway inflammation, which involve many cells and cellular elements (1, 2). Hence, asthma is a heterogeneous condition with distinct clinical phenotypes that in turn may be associated with different types of inflammatory response.

Heterogeneity of airway inflammation has been well documented in adults with persistent asthma of different severity (3, 4) and at exacerbations (5). Although eosinophilic bronchitis is the most characteristic type of inflammation in asthma, this is neither an exclusive feature nor the only type of inflammation observed (6). Sputum cell counts are able to identify eosinophilic, neutrophilic, both eosinophilic and neutrophilic, and paucigranulocytic patterns of airway inflammation in asthma (7). This is relevant because different types of inflammation may respond differently to asthma treatments (8). Interestingly,

the documentation of the heterogeneity of airway inflammation in children is scarce (9, 10).

In addition, there is increasing evidence to suggest that wheezing in childhood represents a heterogeneous condition, with phenotypic expressions associated with different clinical manifestations and risk factors (11). The recent definition of distinct wheeze/asthma phenotypes in childhood has shed light in the natural history of the condition, identifying different groups after the age of 6 years, such as preschool/nonatopic asthma (NAA) and atopic asthma (AA) (12). Although the approach for defining which variables fit the best model explaining these clinical phenotypes may be disputed (13) it seems clear today that they identify both distinct pathophysiological mechanisms and prognosis.

We propose that AA and NAA phenotypes present different types of airway inflammation. We therefore sought to investigate in a nested case-control study, part of a larger cohort followed by our group (14), whether induced sputum could identify the airway inflammatory

characteristics of school-age children with AA and NAA in relation to a control population.

Methods

Subjects

A population of 1011 children attending public schools in a predominantly poor population in Southern Brazilian city was included in the analysis of the ISAAC-phase II study, as described elsewhere (14). A subsample of 90 children of this cohort was randomly selected and equal numbers were assigned to each of the three clinical groups: AA, children with positive answers to both ISAAC questions of 'wheeze in the previous 12 months' and 'asthma ever', and at least one positive skin prick test; NAA, children with positive answers to both ISAAC questions of 'wheeze in the previous 12 months' and 'asthma ever', and negative skin prick tests; and no asthma/no atopy (NANA), children with no asthma or wheeze ever and negative skin prick tests.

The main exclusion criteria were: inability to perform spirometry, an FEV₁ < 75% of predicted, inability to produce induced sputum, gestational age at birth < 37 weeks, use of oral or inhaled steroids in the previous 30 days, a history of cardiovascular or immune deficiency conditions, presence of other chronic respiratory diseases, asthma or rhinitis exacerbations or diagnosis of an acute respiratory infection in the previous month. Parental written informed consent was obtained from all participants. The study was approved by the Human Ethics Committee of the Pontificia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil.

Clinical methods

All participating children answered the ISAAC questionnaire and were skin prick tested for six common aero-allergens (ALK-Abelló, Madrid, Spain), as per the ISAAC = phase II protocol (15). A child was considered atopic if at least one of the tested allergens had a mean wheal diameter ≥ 3 mm, minus the negative control. Spirometry was performed according to the American Thoracic Society recommendations (16) before and 10 min after 200 µg of albuterol inhaled through a spacer; predicted values were obtained from Polgar et al. (17). Sputum was induced with a nebulized 4.5% saline solution according to the modified protocol of Pin et al. (18) and processed as described by Pizzichini et al. (19). The sputum samples were labeled as eosinophilic if sputum eosinophils were 3.0% or more, and noneosinophilic if sputum eosinophils were less than 3.0%.

Statistical methods

Demographic data were summarized using descriptive statistics. Continuous data were summarized by arithmetic mean and standard deviation, or by median and quartiles. Variables with skewed distribution (total cell count and eosinophils %) were log transformed before analysis. ANOVA or chi-squared tests were used for cross-section comparisons between groups. The significance of differences between groups was corrected for multiple comparisons. All tests were two-sided and significance was accepted at 95% level and the analyses were performed on SPSS, version 16.0 (SPSS, Chicago, IL, USA).

Results

Of the 90 selected children, 76 (84.4%) agreed to participate in the study (28 in the AA, 29 in the NAA

and 19 in the control, NANA group). Of these, 55 children (72.4%) provided adequate sputum samples with a good yield index (21 in the AA, 21 in the NAA and 13 in the NANA). There were no clinical or demographic significant differences between included (*n* = 55) and excluded (*n* = 21) children in the study (data not shown).

Characteristics of subjects

Participating children were attending public middle schools. Phenotypic, demographic and spirometric data of the 55 children are shown in Table 1. Subjects of the AA group lived in neighborhoods with better social amenities in town and their mothers had both significantly more years of schooling, and a positive history of asthma when compared with the other two groups. Nonatopic asthmatic children had greater number of siblings when compared with the AAs.

All children presented spirometric values within normal limits. However, prebronchodilator FEV₁ was significantly lower in the asthma groups when compared with the control group. Also the FEV₁/FVC ratio was significantly lower in the AA and NAA groups when compared with the NANA group (Table 1).

Sputum cell counts

Except by sputum eosinophils, total and differential cell counts were similar between groups. Sputum eosinophilia was present in 81% of children with AA. In contrast, 76.2% of children with NAA had noneosinophilic sputum (Table 2). Atopic asthmatic children had signif-

Table 1. Demographic, phenotypic and spirometric characteristics of studied subjects (*n* = 55), as divided in three clinical phenotypes

Characteristics	AA (<i>n</i> = 21)	NAA (<i>n</i> = 21)	NANA (<i>n</i> = 13)
Age in years*	12.9 (0.8)	13.0 (0.7)	13.0 (0.7)
Gender, male, <i>n</i> (%)	12 (57.1)	10 (47.6)	5 (38.5)
Maternal smoking, <i>n</i> (%)	7 (33.3)	6 (28.6)	5 (38.5)
Maternal history of asthma, <i>n</i> (%)	3 (14.3)†	2 (9.5)	1 (7.7)
> 8 years formal maternal schooling, <i>n</i> (%)	4 (19)†	2 (9.5)	1 (7.7)
Affluent neighborhoods, <i>n</i> (%)	3 (14.3)†	2 (9.5)	1 (7.7)
Bronchiolitis event <2 years age, <i>n</i> (%)	3 (14.3)	3 (14.3)	0 (0)‡
> 1 sibling, <i>n</i> (%)	14 (66.7)	19 (90.5)§	10 (76.9)
Spirometry¶			
Pre-BD FEV ₁ , % predicted*	92.6 (5.5)**	93.9 (4.8)	96.8 (3.9)
Pre-FEV ₁ /FVC ratio, %*	81.9 (3.3)††	82.3 (3.8)‡	87.5 (5.2)
Post-BD FEV ₁ , % predicted*	94.6 (3.9)	95.2 (3.9)	97.1 (2.3)

AA, atopic asthma; NAA, nonatopic asthma; NANA, no asthma/no atopy.

*Data expressed as means (SD).

†Significant difference when compared with NAA and NANA, *P* < 0.05.

‡Significant difference when compared with NAA and AA, *P* < 0.05.

§Significant difference when compared with AA and NANA, *P* < 0.05.

¶FEV₁ predicted values (17).

**Significant difference when compared with NANA, *P* < 0.05.

††Significant difference when compared with NANA, *P* < 0.001.

Table 2. Differential cell counts* from induced sputum in the three clinical phenotypes

Characteristics	AA (n = 21)	NAA (n = 21)	NANA (n = 13)
Cell viability, %	86 (64–95)	84 (50–95)	88 (66–93)
TCC† $\times 10^6/\text{mg}$	3.4 (1.2–13.0)	4.0 (1.5–7.6)	2.2 (1.8–8.0)
Neutrophils %	11.0 (7.0–16)	18.0 (7.0–31)‡	13.0 (8.0–22.0)
Eosinophils %	9.0 (0.2–64.0)§	1.0 (0–66.0)	0.5 (0–1.8)
Macrophages %	76.4 (29–86)	78.0 (24–84)	81.0 (74–87)
Lymphocytes %	0.8 (0–4)	1.0 (0–5.0)	0.8 (0–2.0)
Sputum eosinophilia¶, n (%)	17 (81)†	5 (23.8)	0 (0)

AA, atopic asthma; NAA, nonatopic asthma; NANA, no asthma/no atopy.

*Data expressed as median (minimum–maximum).

†Significant difference when compared with NANA, $P < 0.001$.

‡Significant difference when compared with AA, $P < 0.001$.

§Significant difference when compared with NAA and NANA, $P < 0.001$.

¶Eosinophilia = if sputum eosinophils $\geq 3\%$.

icantly higher median proportions (Table 2) of eosinophils than did both NAA children and control. The median absolute number of eosinophils was also significantly higher (3.2×10^5 cells/g) in children with AA than in NAA children (0.24×10^5 cells/g) and the controls (0.1×10^5 cells/g) (Fig. 1A,B). In contrast, the median percentage of neutrophils was significantly higher in children with NAA (Table 2) than in children with AA and controls. Similarly, NAA had significantly higher median absolute number of neutrophils (7.2×10^5 cells/g) than children with AA (3.5×10^5 cells/g) and controls (4.1×10^5 cells/g) (Fig. 2).

Discussion

The findings of this study are novel and unique because there are few studies evaluating the characteristics of

airway inflammation in induced sputum of different clinical asthma phenotypes in childhood as defined by their atopic status. We have shown that airway eosinophilic inflammation is nearly four times more frequent in children with AA than in children with NAA. In contrast, airway inflammation in NAA children is predominantly neutrophilic. This apparent simple stratification based on IS inflammatory cell profile may aid understanding of disease mechanisms and offer a method of monitoring the impact of therapy in different asthma phenotypes.

A recent study from Australia used a slight different approach than ours but its results are in line with our current findings (20). Asthmatic children aged 6–17 years were followed for a mean time of 5 years and classified at the start of the study as eosinophilic or noneosinophilic asthma based on their IS cell profile. Children with eosinophilic asthma were more symptomatic, used more beta-2 agonists, presented lower FEV₁/FVC ratio and were more allergic to pollens when compared with noneosinophilic asthmatics. They were also more likely to be atopic but there was great overlap of positive skin tests in both groups. Our own data show that atopy is associated with more uncontrolled asthma and that NAAs are more likely to have milder disease (14). Both studies thus suggest that there is a close association between AA and eosinophilic inflammation in the airways, leading to more uncontrolled/persistent disease.

Instead, NAA is less likely associated with an eosinophilic sputum profile. Lovett et al. (20) classify these children simply as noneosinophilic, but our data are even more specific, suggesting that there is a higher concentration of neutrophils in the airways of these asthmatics. We have previously shown (14) that the most significant risk factor associated with asthma among nonatopics was a diagnosis of viral bronchiolitis in the first years of life.

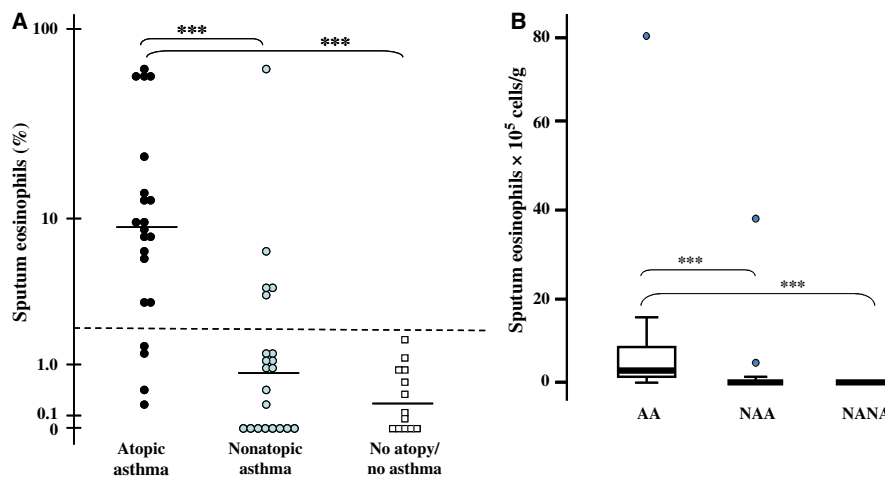


Figure 1. (A) Individual plots of percent sputum eosinophils among atopic asthmatics, nonatopic asthmatics and nonatopic/nonasthmatics. Horizontal bars represent the median values; *** P -values < 0.0001 for comparisons between groups. (B) Box plots of absolute numbers of eosinophils per gram of sputum. Horizontal bars represent the median values. *** P -values < 0.0001 for comparisons between groups. In comparison with nonatopic asthmatics and nonatopic nonasthmatic subjects, those with atopic asthma had significantly higher absolute numbers of sputum eosinophils.

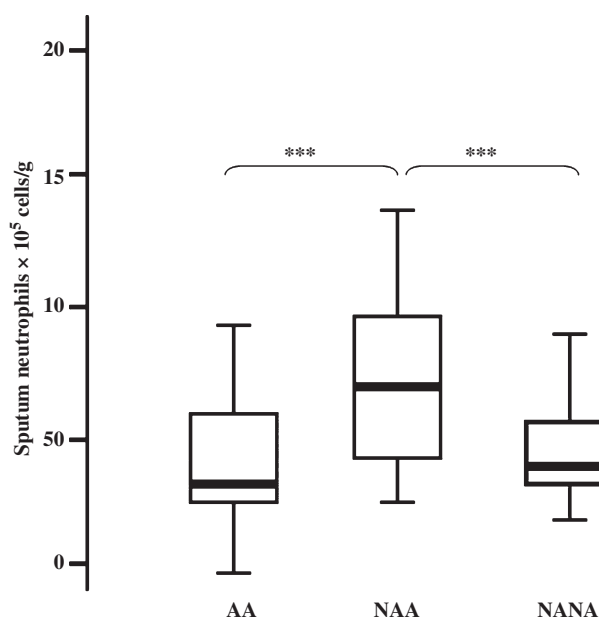


Figure 2. Box plots of absolute numbers of neutrophils per gram of sputum. Horizontal bars represent the median values. ****P*-values < 0.0001 for comparisons between groups. Children with nonatopic asthma (NAA) had significantly higher absolute numbers of sputum neutrophils than atopic asthmatics (AA) and nonatopic nonasthmatic subjects (NANA).

With the current knowledge, it is reasonable to hypothesize that there is an association between early life viral respiratory infections (either by Respiratory Syncytial Virus (RSV) or Rhinovirus) and persistent wheeze/asthma although it is not possible at this point to have a definitive explanatory hypothesis (21). Yet, there is consistent data showing that virally induced airway inflammation elicits a predominant neutrophilic acute response and that in some clinical situations after a primary viral insult a persistent and long lasting airway inflammatory response may result (22). Experimental data suggest that chronic neutrophilic inflammation (e.g. as in chronic smoking or cystic fibrosis) may reduce Surfactant Protein D levels, which normally contributes to the early innate immune response to viral infections, thus leading to a greater predisposition to these infections. This is in contrast with acute inflammation, as happens in the early phase of the viral infection, which promotes neutrophil-mediated viral clearance (23).

Even though our findings point in the direction of a dichotomous inflammatory profile for AA and NAA, the subjects from this study did not show very high neutrophil levels. Despite of the statistical significance, this finding may not indicate clinical significance. There is no established validation of 'normal' cutoff values for neutrophils in induced sputum, especially among children. These concentrations are known to present great variability and can range from 17% to almost 80% (24). This variability may be influenced by different factors that affect neutrophilic distribution, such as

environmental pollution, concurrent respiratory infections, asthma exacerbation and patients' age (24, 25).

Thomas et al. (24) have shown that the normal concentration of neutrophils in induced sputum is closely associated with age; the lower the age, the lower the concentration of these cells. Adults with a mean age of 60 years have mean neutrophil concentrations of 68.5% (SD 20.6%), while young adults at age 18 presented lower concentrations with mean of 26.9% (SD 19.8%). This concept may suggest that a median neutrophil proportion of 13% (as found in our AA group) or 18% (in the NAA) such as found in our children at age 10 years may not have clinical significance but are valid for a comparison between groups.

The expression of noneosinophilic inflammation is associated with an increased neutrophil presence in the bronchial mucosa, similar to what is observed in occupational asthma (25). It is also related to external factors (e.g. air pollution, bacterial endotoxins and viral aggressions) that stimulate the release of pro-inflammatory cytokines (such as IL-8) responsible for the infiltration and activation of neutrophils in the airways (25). Neutrophil chemoattractant cytokine levels such as IL-8 are closely associated with the variation in the proportion and number of neutrophils in the lower airways of normal children (26). A study by Simpson and collaborators goes in the same direction of our current findings suggesting that neutrophils also play a major role in the characterization of airway inflammation in asthma (27, 28).

One possible criticism of our findings would be misclassification bias related to the definition of asthma phenotypes by questionnaire and skin test responses. In fact, it was reassuring to observe that our AA group consisted of children who lived in better neighborhoods of town (i.e. overall better sanitary conditions), their mothers had both better schooling and were more likely to have a history of asthma, when compared with the other groups. In our previous study where we analyzed the entire cohort, we have shown that children presenting the classic, AA were more likely to have higher Socio-Economic Standard (SES) and uncontrolled disease (14). Instead, NAAs had greater number of siblings, thus, more likely to be poor and to be at greater risk for infections. These findings have been reproduced in other communities (15) and are consistent with the paradigm of the hygiene hypothesis.

Yet another possible criticism would be the small number of subjects included in this study. If anything our findings of a significant difference on cell-type expression in sputum between AA and NAA groups in such a small number of subjects reinforce the fact that these variables have great discriminatory value. Nevertheless, we agree that a larger sample size would help in better defining these differences, especially when compared with a normal control group.

Our results not only reinforce the concept of distinct asthma phenotypes in childhood, but also offer new information on a practical noninvasive monitoring tool

for evaluating asthma and the impact of current therapeutic options. It also adds further strength to the concept that atopy when associated with asthma leads to a more persistent and, probably, a more severe form of disease but it does not explain all asthma. The identifi-

cation of forms of asthma not associated to either atopic markers or predominant eosinophilic inflammation may help us better understand other underlying mechanisms that would maintain asthma symptoms through a major neutrophilic pathway.

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