



Induced sputum in children and adolescents with asthma: safety, clinical applicability and inflammatory cells aspects in stable patients and during exacerbation

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Abstract

Objectives: To determine the safety of sputum induction in asthmatic children and adolescents, to characterize sputum inflammatory cells while clinically stable and during exacerbation and to correlate sputum inflammatory cells with peripheral blood eosinophils, serum IgE and the degree of bronchial obstruction.

Methods: Ninety-six asthmatic patients aged 6 to 18 years were recruited for the present cross-sectional study. Spirometry was performed before and after administration of a bronchodilator. Sputum was collected spontaneously or after induction by the inhalation of saline solution at increasing concentrations. Blood samples were obtained for serum IgE and eosinophil quantification.

Results: Sputum samples adequate for analysis were obtained from 68 (70.8%) of the patients recruited. No relevant bronchoconstriction was observed during induction. The presence of a larger number of eosinophils in sputum did not correlate with more clinically severe asthma. No correlation was observed between the degree of bronchial obstruction, measured based on FEV₁, and inflammatory cells in sputum, peripheral blood eosinophils or serum IgE. Larger numbers of neutrophils were observed in the asthma exacerbation group ($p < 0.05$).

Conclusions: Sputum induction was found to be a safe procedure for obtaining clinical samples from children and adolescents even during exacerbations, allowing for clinical and functional limitations. The 67% induction success rate was considered satisfactory. In this group of patients, receiving inhaled corticosteroids, eosinophil quantification did not distinguish between the clinical and functional severity of asthma and was independent of the degree of airway obstruction. A proportional predominance of neutrophils was observed in the sputum of patients with asthma exacerbation.

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Introduction

Asthma is a chronic inflammatory disease of the airways that causes variable airflow obstruction and bronchial hyperresponsiveness.¹ Several different cell types are involved in this inflammatory process, in particular lymphocytes, neutrophils, labrocytes, eosinophils and epithelial cells. Eosinophils produce cytotoxic proteins and leukotrienes that are capable of damaging the airway epithelium are also a factor in continuing cell recruitment.² Neutrophils appear to play a role more relevant to

exacerbation, nighttime asthma, hard to treat asthma and to severe asthma refractive to corticosteroids and it is suggested that they participate in airway remodeling.³

The intensity of the inflammatory process can be assessed either directly or indirectly. Bronchial biopsy and broncho-alveolar lavage have been used to analyze cell response patterns in relation to clinical severity, cell subtypes, inflammatory cell activation and expression and the production of cytokines and of inflammatory response mediators, mediated by eosinophils.⁴ However, these are invasive procedures and cannot be applied to routine monitoring of the inflammatory process, particularly in children. Over recent years markers of inflammatory activity have been sought in asthmatics, employing less invasive techniques such as measuring nitric oxide in exhaled air and sputum cell typing.

Sputum can either be obtained spontaneously or by induction with inhaled hypertonic saline solution (HSS).^{4,5} The induced sputum test has been shown to be a safe method, with the potential to characterize the intensity and development of the inflammatory process. However, the peculiarities of the cytology at different degrees of asthma intensity and the differences between stable and exacerbated states have not been fully evaluated in children.

The objectives of this study were: 1) to verify the safety and clinical applicability of sputum induction in children and adolescents with asthma; 2) to characterize the cellular characteristics of sputum in a group of patients with asthma of varying levels of severity, during a clinically stable period and in patients in exacerbated states; 3) to correlate sputum cellular characteristics with degree of bronchial obstruction, the number of eosinophils in peripheral blood and total serum IgE assay.

Patients and methods

Ninety-six children and adolescents with asthma diagnoses¹ were recruited. They were regular patients at the Children's Institute's Pneumology and Immunology Clinics at the *Hospital das Clínicas of Universidade de São Paulo* Medical Faculty and were sent to the laboratory for pulmonary function tests to undergo routine spirometry. Patients aged 6 to 18 years were enrolled with varying levels of clinical severity¹ and capable of performing spirometry. Patients were excluded if they had repeated pulmonary infections, persistent radiological abnormalities or any datum suggestive of other lung disease. Patients were defined as stable if they had no disease exacerbation and had been free of infectious episodes for the four weeks prior to testing. Patients were defined as suffering asthma exacerbation if they attended for spirometry, but, according to parents and/or clinical evaluation, had clinical signs of disease exacerbation such as coughing and/or dyspnea and/or wheezing. In order to classify asthma severity a brief questionnaire was applied in order to obtain clinical data (symptoms and current treatments) and pre-bronchodilator, first-second forced expiratory volume (FEV₁) was used.

Spirometry was performed in accordance with international technical recommendations⁶ using a water-seal spirometer (Warren Collins, Inc, MA, USA). The values obtained for forced vital capacity (FVC) and FEV₁ were expressed as percentages of expected values according to the equations proposed by Polgar & Promadhat.⁷ Spirometry was repeated fifteen minutes after the administration of 400 µg of salbutamol via aerosol. Bronchodilator (BD) response was defined as significant if there was an increase in FEV₁ equal to or greater than 7% of the expected value.⁸ Before base and post-BD spirometry were performed, peak expiratory flow (PEF) was measured using a peak flow meter (Peak Flow Meter Mini- Wright, Clement Clark International).

Sputum induction and processing

After spirometry had been performed the patients were requested to cough and spit into a sterile polypropylene pot. If a sample that was sufficient for analysis was obtained then induction was not performed. Sputum was induced with HSS in accordance with methodology described by Pizzichini,⁹ modifying only the nebulizer employed. A Proneb compressor was used with a Pari nebulizer with a mouthpiece fitted with an expiratory valve, flow rate of 0.87 ml/min and an approximate particle emission of 6 µg. Stable patients whose FEV₁ was equal to or greater than 50% of expected were given HSS nebulization with a nose clip, for 10 minutes at each of the concentrations 3, 4 and 5%. The maximum period of nebulization was 30 minutes in order to collect at least 0.5 ml of sputum. Stable patients whose FEV₁ was < 50% of expected and those in exacerbation were given nebulization with just saline solution at physiological concentration (PSS). Measurements were taken of PEF and pulmonary auscultation was performed immediately after each nebulization. In the presence of dyspnea, wheezing and/or a drop in PEF > 20% of base, the induction procedure was suspended and 200 µg of inhaled salbutamol was administered. If PEF dropped by 10 to 20%, further nebulization was not contra-indicated, but the same saline concentration was maintained. Variations of PEF of less than 10% were acceptable for nebulization to be continued at the next HSS concentration. After each nebulization, the patients were requested to wipe their noses and rinse their mouths with water in order to avoid contamination with saliva and then cough and spit into the pot.¹⁰ The pot containing the sputum was kept in a polystyrene box with ice and processed within 2 hours of collection. All procedures were followed by one of the researching doctors who was available at the laboratory, in case material or medication became urgently necessary.

The material collected was placed in a Petri dish and sputum was separated from saliva using a polypropylene pipette. The sample was processed using the methodology described by Pizzichini.⁹ Viability and total cell counts were verified in a Neubauer chamber and under optical microscope accepting a maximum of 50% of non-viable cells and 80% of squamous cells. Cytospins were prepared using a Shandom

III centrifuge (Shandom Southern Instruments, Sewickley, PA, USA) centrifuging at 450 rpm for 6 minutes. Slides were stained with Leishman.¹¹ One or two slides were read until a count of 400 non-squamous and non-metachromatic cells and was reached and differential data was given in percentages.

On the same day, immediately after the induction procedure, a blood sample was taken from patients from whom a sputum sample of at least 0.5 ml had been obtained in order to assay total serum IgE and prepare a differential leukocyte count.

The research project was approved by the Ethics Committee for Research Project Analysis at the *Hospital das Clínicas of Universidade de São Paulo* Medical Faculty. Patients and parents or guardians were informed of and consented to participate in the study.

Statistical analysis

Depending on the variable in question, descriptive analysis employed mean and standard deviation or median and percentiles. Spearman or Pearson correlation was used depending on data distribution, defining coefficients (r) greater than 0.50 and p less than 0.01 as significant.

Results

Ninety-six asthmatic patients in the age group of 6 to 18 years were recruited. From 10 patients sputum was obtained spontaneously, the remaining 86 patients were subjected to the sputum induction process. In 28 cases the sputum induction failed to obtain material or the sample was inadequate (success rate = 67%). The study group, therefore, was made up of 68 patients.

The mean age was 10.5 years, with 48 patients (70.5%) between 6 and 11 years old. With respect of sex, 27 (39.7%) were female and 41 (60.2%) were male (proportion M:F = 1:1.5). Stable patients were grouped by asthma severity: 11 patients (18.3%) had mild asthma (intermittent or persistent); 20 had moderate asthma (33%) and 29 severe asthma (48.3%). The group in asthma exacerbation included eight patients. Clinical and functional characteristics for the groups are given in Table 1. The group of patients with severe asthma exhibited FEV₁ values that were reduced in relation to the mild and moderate asthma groups (p < 0.001 and p = 0.003, respectively). The group of patients suffering asthma exacerbation also exhibited reduced FEV₁ when compared with the mild and moderate asthma groups (p < 0.001). A response to BD was observed with greater frequency in the severe asthma group (p < 0.05).

Table 1 - Characteristics of children and teenagers with asthma according to severity,¹ functional and laboratory parameters

	Mild *	Moderate	Severe	Asthma exacerbation	p
Patients (n)	11	20	29	8	—
Sex F/M	4F/7M	8F/12M	13F/16M	2F/6M	—
Age (years), mean±SD	9.8±2.6	9.9±2.2	11.4±3	10±1.6	—
Using inhaled corticosteroids	3 (27%)	13 (65%)	24 (83%)	7 (87%)	—
Pre FEV ₁ %, mean±SD	102.4±8.4	90.5±13.5	72.4±13.7 ‡ §	70±16.2 † ‡	† p < 0.001 ‡ p < 0.001 § p < 0.001 ‡ p = 0.003
FEV ₁ /FVC%, mean±SD	88.4±5.6	83.2±5.5	73.3±1.6 ‡ §	77.8±10.9	§ p < 0.05 ‡ p < 0.05
n, BD response > 7%	1/11 (9%)	6/20 (30%)	20/29 (69%) §	6/8 (75%)	§ p < 0.05
IgE UI/ml M (min-max)	1,037 (331-3,428)	771 (259-14,940)	989 (29-11,110)	365 (30-1,833)	NS
Blood Eos M (min-max)	546 (103-1,003)	616 (192-1,278)	510 (82-1,760)	431 (237-620)	NS

SD = standard deviation; FEV = forced expiratory volume; FVC = forced vital capacity; BD = bronchodilator; IgE = serum immunoglobulin E; Eos = eosinophils;

M = minimum and maximum values median; NS = non significant.

* The group with mild asthma comprises patients with intermittent or persistent asthma due to FEV₁ > 80%.

† p < 0.001 between the groups with exacerbation and with mild asthma.

‡ p < 0.001 between the groups with exacerbation and with moderate asthma.

§ p < 0.001 between the groups with severe and mild asthma.

§ FEV₁/FVC § p < 0.05 between the groups with severe and mild asthma.

‡ p < 0.05 between the groups with severe and moderate asthma.

Total serum IgE assay was elevated for age in 62 patients (91%) and 47 (70%) presented peripheral eosinophilia above 5%. These results did not correlate with asthma severity.

The mean inhalation period for sputum induction was 18 ± 7.8 minutes. There was a drop in PEF with respect of base value of up to 10% in five patients (7.35%), from 10 to 20% in three (4.4%) and >20% in two (3%). In these patients who met the criteria for ceasing the sputum induction procedure, bronchoconstriction was promptly reversed with BD and there were no detectable clinical complications.

The volume of sputum obtained varied from 0.5 to 5 ml. The mean percentage of viable cells was $74 (\pm 16)$ and the mean percentage of squamous cells was $21.62 (\pm 22.2)$.

Table 2 gives the total cell counts and differentiated cellular characteristics for the various patient groups. There were no differences between mean eosinophil percentages for the three groups of patients with stable asthma. Stable patients using inhaled corticosteroids exhibited a mean

eosinophil percentage of 10.48%, while, for those not on corticoid therapy, this figure was 9.51% ($p > 0.05$) (Table 3). Great variability was observed in eosinophil percentages across the entire study population and 60% of the stable patients received inhaled corticosteroid, but still presented eosinophil percentages in sputum above 2.5%.

A predominance of neutrophils was observed for the group of patients with asthma in exacerbation when compared with the remaining groups ($p < 0.05$). The variations in eosinophil and neutrophil percentages across the different groups are best viewed in Figures 1 and 2, respectively.

Figure 3 shows the dispersal in correlation between percentage of eosinophils in sputum and values for FEV_1 for all patients in the study. There was no correlation between eosinophil counts for sputum and the degree of bronchial obstruction as measured by pulmonary function ($r = 0.118$ and $p = 0.336$). The same correlation analysis was performed for the other cell counts (lymphocytes, macrophages and neutrophils) and the results were similar.

Table 2 - Sputum cell typing according to severity. Total and differential cell count, values expressed in mean \pm SD

	Mild	Moderate	Severe	Exacerbation	p
Cel x10 ⁶ /ml	2.2 \pm 2.4	1.6 \pm 1.7	1.4 \pm 1.4	5 \pm 4	NS
% eosinophils	4.9 \pm 4.8	8.4 \pm 13.5	12.6 \pm 21.1	0.7 \pm 1	NS
% neutrophils	35.7 \pm 23 *	47.2 \pm 24.1 *	44.8 \pm 29.8 *	80.8 \pm 13.2 *	* p < 0.05
% macrophages	48.8 \pm 20.9 †	37.1 \pm 23	38.4 \pm 29.6	13.6 \pm 9.3 †	† p < 0.05
% lymphocytes	1.28 \pm 2.5	1.14 \pm 2.9	0.32 \pm 0.8	0	NS
% epithelial cells	0.8 \pm 0.2	2.1 \pm 0	0.28 \pm 0	1.63 \pm 0	NS

SD = standard deviation; Cel = cells; NS = non significant.

* p < 0.05 for the group with exacerbation as compared to the groups with mild, moderate and severe asthma.

† * p < 0.05 for the group with exacerbation as compared to the group with mild asthma.

Table 3 - Sputum cell type in severity groups according to inhaled corticoid therapy (IC). Values expressed in mean \pm SD or median (p25-p75)

	Mild		Moderate		Severe		p
	With IC	Without IC	With IC	Without IC	With IC	Without IC	
% eosinophils	6.97 \pm 5.5	3.74 \pm 5.3	2.6 (1.1-9.3)	3.5 (0-6.9)	5 (0.6-13.1)	2.5 (0-27)	NS
% neutrophils	34.5 \pm 25.8	36.8 \pm 28.8	47.8 \pm 21.6	48.9 \pm 31.9	43.6 \pm 30.5	52.5 \pm 27.6	NS

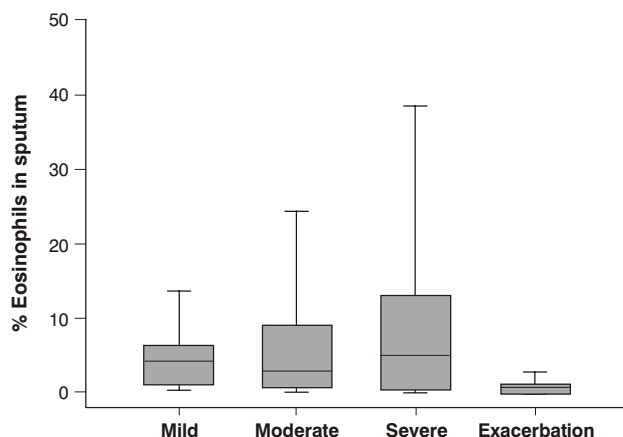


Figure 1 - Distribution of eosinophils (%) in groups of patients with asthma of varying levels of severity
 $p = NS$

Discussion

This study described the cellular characteristics of sputum, obtained either spontaneously or after induction with HSS, from children and adolescents with asthma of varying degrees of severity, either during a stable phase of the disease or during an exacerbation.

Since the induction technique was added to the routine of the pulmonary function laboratory at the Children’s Institute with the realization of this study, the initial objective was to verify if it was practicable and safe with modifications made necessary by our situation. Sputum induction was successful in 67.4% of attempts. Studies involving similar

patient samples report success rates of 56%,¹² 84%¹³ and 95%.¹⁴ According to Paggiaro,¹⁵ obtaining adequate sputum samples is more related to individual patient characteristics than associated with technical factors during collection of the material.

The type of nebulizer employed can have an influence on the success of induction, and it is the De Vilbiss ultrasonic nebulizer that produces the greatest flows and greatest volumes of sputum. ON the other hand, low flow rate nebulizers (below 1 ml/minute) cause less respiratory discomfort than those with high flow rates, which may be more suitable for use with children.^{16,17} The present

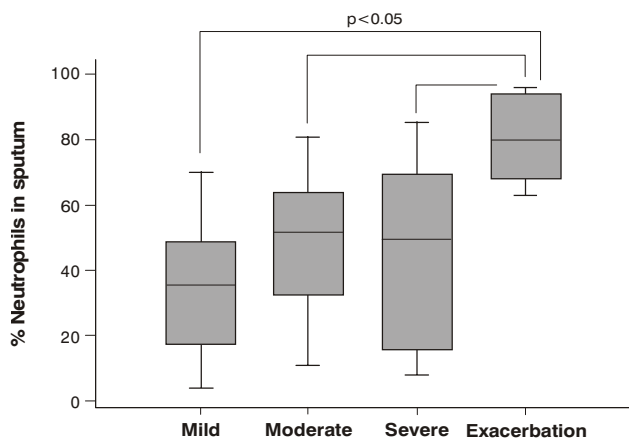


Figure 2 - Distribution of neutrophils (%) in induced sputum in groups of patients with asthma of varying levels of severity

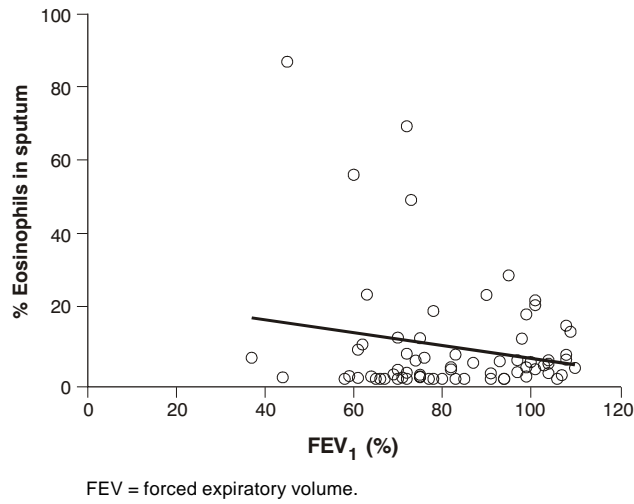


Figure 3 - Dispersal in correlation between percentage of eosinophils in sputum and values for FEV₁ (%) of 68 children with asthma of varying levels of severity $r = 0.118$ and $p = 0.336$ for all patients in the study

study employed a Proneb-Pari, low flow rate nebulizer, available at the laboratory and better adapted to the age group being studied without negatively impacting the procedure's success.

There is no difference in cell counts when nebulization is performed with PSS or HSS in order to induce sputum, irrespective of asthma severity.¹⁶ However, induction is more successful with HSS than with PSS¹⁷ Nevertheless PSS is safer and better tolerated by patients with severe asthma.¹⁸ In this study we performed induction with either PSS or HSS depending upon the clinical and functional severity of each patient. It should be pointed out that using this method there were no complications related to the induction process.

In a multicenter study, Fahy *et al.*¹⁹ observed that certain patients were more hyper-reactive to HSS, irrespective of clinical or functional severity, and that they suffered bronchospasm even when BD had been giving in advance, reporting drops in FEV₁ of between 40 and 60% around 4 minutes after the start of induction with HSS. In the current study it was observed that 6.8% of the total patient sample had a drop in PEF. In our study, just two patients exhibited bronchoconstriction, recorded as a 20% drop in PEF, and this was promptly reversed with BD with no need for hospitalization or referral to the emergency department.

The mean time required to induce sputum was 18 minutes, 75% of the patients produced sputum after 10 to 20 minutes and 21% required 30 minutes. International

consensus is that 15 to 20 minutes is adequate for induction. Different cellular compartments of the airways are sampled depending on the total period of induction. During the initial minutes components of the central airway are sampled with predominance of neutrophils and during the final minutes the distal airways are sampled together with the alveolar compartment giving a predominance of macrophages.^{20,21}

The quality of the material analyzed depends on the time passed before the start of sputum processing, on the number of squamous cells present in the sample and on the quantity of inflammatory cells analyzed.¹⁶ Ideally, it is suggested that sputum should be processed within 2 hours of collection.²² It is recommended that saliva be separated, since the lower the number of squamous cells the better the quality of the slides and the easier it is to identify inflammatory cells. A minimum of four hundred inflammatory cells should be counted and it is recommended that up to five slides are read.^{23,24} Cell viability below 50% and contamination with squamous cells above 20% make the method less reproducible, but sputum samples are acceptable with up to 80% of squamous cells.^{4,19} Despite all the care that was taken in our study with collection and separation of material, it was necessary to read more than one slide in 16% of the samples analyzed because the initial slide contained between 50 and 80% of squamous cells. This detail did not interfere with the quality of the results, but it did extend the processing time even further. The processing time, around 60 to 90 minutes per sputum sample, together with the time taken to induce sputum production, limits the number of examinations that can be analyzed in a single day.

Attempts could be made to store the material at 4 °C for up to 9 hours after collection, without altering the total or differential cell counts.²² The availability of time and specialized personnel is something that must be taken into account when the decision is being made whether to institute this technique as routine.

Cai *et al.* demonstrated that the maximum percentage of eosinophils in the sputum of normal children is 2.5%.²⁵ Furthermore, eosinophils in sputum is a characteristic of asthma in the pediatric population, can be related with clinical severity of the disease and reduces after corticoid therapy.²⁶ It is estimated that more than 80% of asthmatics who have not previously used corticosteroids and more than 50% of those who do use inhaled corticoid therapy, may have above normal eosinophil levels in sputum.²⁷ In our study, in common with what was described by Gibson,²⁸ we observed that 60% of the stable patients used inhaled corticoid therapy and had eosinophil percentages above 2.5% in sputum.

There is no well-founded recommendation of the clinical conduct to be employed in this situation, when there is clinical and functional stability, but markers of inflammation are demonstrated in the airways. Green *et al.* proposed a strategy for asthma treatment in which disease control was based on maintaining eosinophil counts in sputum below 3%. When the treatment group was compared with patients treated according to current recommendations – clinical and functional monitoring – it was found to have lower numbers of exacerbations and hospitalizations, lower oral steroids requirements and lower global treatment costs.²⁹ The discussion on the best form of monitoring asthma treatment progress and how to decide if the disease is under control is very current. Strategies based on markers of inflammation in the airways require serial measurements and, in this case, serial sputum cell counts. When employing a technique with a success rate of around 70% for the pediatric population, serious limitations can come up with respect of regularly obtaining the data needed for patient follow-up.

While a progressive increase in the percentage of eosinophils in sputum as asthma severity increased was observed, this difference was not significant between the groups. This may be explained, in part, by the fact that the majority of the children studied had used inhaled corticosteroids, at medium to high dosages, for periods of more than 3 months. Previous studies have demonstrated that this length of treatment at these doses is sufficient to reduce the number of eosinophils in sputum significantly and that reducing the dose or suspending the treatment can reactivate an increase in eosinophils.³⁰ Bartoli *et al.* described the cellular characteristics of sputum in a larger group of 223 asthmatic patients of varying severity and 14 controls, and noted that patients with persistent mild and moderate asthma did not differ in terms of eosinophil counts in sputum, irrespective of regular treatment with inhaled corticosteroids. In this study, as expected, the percentage of eosinophils in sputum adequately separated asthmatic patients from controls.³¹

There are published reports that suggest that cell counts and other inflammatory markers present in sputum are related to asthma severity.²⁶ In contrast, the results of well-conducted studies contest this claim and the true role of sputum cell counts in discriminating asthma severity remains controversial.³² Indeed, the international working group on induced sputum itself comments that there is a weak relationship between asthma severity defined by pulmonary function or symptoms and eosinophil counts in sputum and that further studies are needed to define the relationship between airway inflammation, symptoms and response to corticosteroids in asthmatic patients.³³

In our study, despite the significant reduction in FEV₁ means for the group of severe asthmatics, there was no significant correlation between the percentage of eosinophils in sputum and the degree of bronchial obstruction. It is important to point out that we used the classification of patient clinical severity based not just on clinical and functional criteria, but also taking account of previous treatment, in accordance with international recommendations.¹ Therefore, certain patients were defined as severe asthmatics because they were on elevated doses of inhaled corticosteroid, despite exhibiting little obstruction according to FEV₁. Pin *et al.*,⁴ studying adults and adolescents, found a significant correlation between the percentage of eosinophils in sputum and the degree of obstruction. Gibson *et al.*, in a more recent publication, studied 146 children with stable asthma of different levels of severity and did not observe this correlation, nor a correlation between the percentage of eosinophils in sputum and bronchial responsiveness.³² The authors explained this by the fact that 94.5% of the children had FEV₁ above 80% of the expected value and, therefore had no detectable obstruction despite having different levels of asthma severity according to clinical criteria. In our study 53.33% had FEV₁ above 80%. This leads to the conclusion that each different method provides different information on the condition of the disease of the patient and are complementary in the characterization of different features of the inflammatory process in asthma.⁵

There were no correlations between the findings for peripheral blood, eosinophils and serum IgE, and the percentage of eosinophils in sputum. This result can be explained in part by the great variability of these parameters for each group. Published data on this issue suggested that eosinophils in sputum were the most accurate marker of inflammation of the airways, since eosinophils in peripheral blood can be elevated in other conditions such as rhinitis and eczema and are not related with asthma severity.²

On the subject of asthma exacerbation, there is consensus on the important increase in total cell counts,^{17,34} probably due to the increased influx of inflammatory cells and consequent increased shedding of the epithelium. This increase in the total number of cells was also observed in our study, from two to four times that of the stable groups, although this difference was not statistically significant, probably due to the small number of patients in exacerbation. Gibson *et al.* studied the cellular characteristics of the sputum of children in crisis and found three distinct patterns:

with eosinophils, with eosinophils and neutrophils and the third non-eosinophilic. They also reported that the group with the greatest percentage of eosinophils was that with no anti-inflammatory treatment, the opposite was true of those using oral corticosteroids and a third of the children in crisis had increased neutrophils.³⁴ Viruses are potent at inducing neutrophilic response and are a frequent cause of asthma exacerbations in children. In the exacerbation group the highest neutrophil levels were observed with respect of all of the stable asthma groups ($p = 0.05$). However, etiologic investigation for respiratory viruses was not performed since this was not the initial objective in evaluating this group of patients.

The procedure of sputum induction, initially described for adults, using saline solution inhaled at increasing concentrations was shown to be a safe method for obtaining clinical samples from children and adolescents, even during exacerbations, taking clinical and functional limitations into account. An adequate sample for analysis was obtained in the majority of cases and the induction success rate of 67% is comparable with published data. Eosinophil in sputum counts were not capable of discriminating clinical and functional severity and of asthma, although the study group was being treated with inhaled corticosteroids. The sputum of patients in asthma exacerbation had, proportionally, a predominance of neutrophils.

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