

ORIGINAL ARTICLE

AIRWAY DISEASES

Morphological changes in eosinophils are reliable markers of the severity of an acute asthma exacerbation in children

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Abstract

Background: Early identification of the severity of asthma exacerbation would be helpful for the management of patients. We aimed to evaluate the correlation of morphological change in activated eosinophils and the severity of an asthma exacerbation.

Methods: Blood was collected from 55 asthmatic children: 40 of whom were having an exacerbation, 15 symptom-free, and 15 healthy controls. The percentage of eosinophils with morphological changes (emission of single or multiple pseudopods, presence of cytoplasmic vacuoles, releasing a small, moderate, or large quantity of granules, spreading, eosinophil death, and presence of cluster of free eosinophil granules) was quantified after the adherence to a slide and compared using the Mann–Whitney test. The correlation between the severity of the asthma exacerbation and the percentage changed eosinophils was tested with Spearman's correlation.

Results: The proportion of activated eosinophils was higher in asthmatic symptom-free children than in the control group, and acute asthma exacerbation produced an additional increase in eosinophil activation ($P < 0.01$). More significantly increased morphological changes were emissions of multiple pseudopods, presence of cytoplasmic vacuoles, spreading, and presence of a cluster of free eosinophil granules ($P < 0.001$). The following were correlated with the severity of an asthma exacerbation: $\geq 14\%$ of eosinophils emitting single pseudopod, 8% emitting multiple pseudopods, 17% with vacuoles, 28% eosinophils releasing a large quantity of granules, and 66% of spread eosinophils.

Conclusions: Quantifying the morphological changes in eosinophils is a feasible, easy, and reliable manner to identify the severity of an asthma exacerbation and therefore might improve the clinical management of asthmatic children.

Asthma is an important clinical condition worldwide, as there are an estimated 300 million affected individuals. Asthma exacerbations pose the greatest risk to asthmatic patients and incur the greatest asthma-related treatment costs for healthcare systems (1). Decisions for the management of asthma have traditionally been clinically and functionally based upon assessing the patient's symptoms and respiratory function (2, 3).

An increased number of eosinophils in the peripheral blood and airway secretions is a characteristic feature of asthma, and the presence of eosinophils has been correlated with the disease severity and bronchial hyper-responsiveness (4). The

deleterious role thought to be played by eosinophils in asthma is linked to their ability to secrete various inflammatory substances, mainly toxic proteins and lipid mediators, into body tissues. This ability is a particular feature of activated eosinophils, which have undergone numerous metabolic, functional, and phenotypic changes from their resting state (5).

In this context, eosinophils are thought to have an important proinflammatory role in the pathogenesis of asthma (6, 7), and their mediators are consistently identified in asthmatic but not in healthy lungs. However, blood eosinophils are an insensitive and imprecise marker of tissue inflammatory

responses (8). Eosinophils are most likely associated with asthma exacerbations, and sputum eosinophilia develops several weeks before the onset of an exacerbation (9). The proportion of eosinophils in the sputum is a more accurate marker of asthmatic airway inflammation than the proportion of eosinophils in the blood (10). Examining the sputum for eosinophils is an excellent tool to assess for inflammation. However, sputum induction can be challenging for young children (11), and blood eosinophils are not a reliable marker. Therefore, we hypothesize that parameters reflecting not only the amount of eosinophils but also their state of activation in the peripheral blood could be an easily obtainable objective marker for the severity of the disease, particularly in acute exacerbations. This work aimed to evaluate whether morphological changes associated with the activation of eosinophils in the peripheral blood can distinguish asymptomatic asthma from acute asthmatic episodes and whether these changes correlate with the severity of acute asthma exacerbations in children.

Methods

Study groups

One author (SMB-M) sequentially and unbiasedly selected and screened 70 children seen in the emergency care unit or the asthma outpatient clinic at Brasília Asa Sul Regional Hospital for enrollment in this study. Peripheral blood (1 ml) was collected from the children, who were divided into the following three groups: a control group, composed of 15 healthy children (five girls and 10 boys; 4–13 years) without the disease and without a personal or familial history of allergy; a group of 15 outpatient asthmatic children with a variable disease severity (three girls and 12 boys; 2–12 years) from whom blood was collected during symptom-free periods between acute asthma exacerbations; and a group of 40 asthmatic children with clinical manifestations of an acute asthma exacerbation (14 girls and 26 boys; 2–13 years) who were seen at the emergency room. There were no statistically significant differences in age ($P = 0.10$) or gender ($P = 0.55$) among the three groups examined.

One author (SMB-M), who is specialized in allergy, conducted a clinical evaluation of all control and asthmatic children. At the time of the clinical examination, peripheral blood was collected to assess for morphological changes.

Asymptomatic asthma and an acute asthma exacerbation were defined according to the Brazilian Consensus for Asthma (12). The exclusion criteria were as follows: children <2 years, any other clinically significant pulmonary disease or other disease, those who presented with any condition that might alter the function of the immune system, and children taking medications.

The disease was classified as intermittent and mild, moderate or severe persistent asthma by the frequency of the symptoms, presence of nocturnal asthma, frequency of acute exacerbations, medications required for control, physical activity limitations, or altered pulmonary functioning, according to the Brazilian Consensus for Asthma (12).

In the group of asymptomatic children, 12 (80%) had intermittent asthma, 2 (13%) had mild asthma, and 1 (7%) had moderate persistent asthma. The group of children with an acute asthma exacerbation included 30 (75%) with intermittent asthma, 5 (12.5%) with mild asthma, 2 (5%) with moderate asthma, and 3 (7.5%) with severe asthma. In the group with an acute asthma exacerbation, the severity of the acute exacerbation was classified as mild/moderate in 23 (57.5%) children, severe in 13 (32.5%) children, and very severe in 4 (10%) children.

The following are the median values (extremes) of some clinical variables of those patients with an acute asthma exacerbation: pulse rate of 117 (78–168) bpm, respiratory rate 34 (16–60) ripm, Sat O₂ 96.5% (79–99%), PaO₂ 51 (50–55) mmHg, and PaCO₂ 55 (50–62) mmHg. The PaO₂ and PaCO₂ were determined only for children with a severe acute exacerbation.

Table 1 explains the assessment of the asthma exacerbation severity and the index score according to the clinical parameters (13). The final score for each child was obtained by calculating the sum of the punctuation achieved during the examination.

Table 1 Score of severity of asthma acute exacerbation

Variable	Condition	Score
General clinical status	Normal	1
	Cyanosis, Exhaustion	3
Alertness	Normal	1
	Agitated, Drowsy, Confused	3
Dyspnea	Absent or Mild	1
	Moderate	2
	Severe	3
Talks in	Sentences	1
	Phrases, Shorter cry, Difficult feeding	2
	Words	3
Use of accessory muscles	Not	1
	Mild, Moderate	2
	Marked	3
Localize or diffuse wheezing	Bass	1
	Loud	2
	Reduced vesicular murmur	3
Respiratory rate (ripm)	Normal	1
	Increased	2
Pulse rate (bpm)	<110	1
	110–140	2
	>140 or Bradycardia	3
SaO ₂ (%) (breathing room air)	>95%	1
	91–95%	2
	≤ 90%	3
PaO ₂ (mmHg)* (breathing room air)	Normal	1
	>60 mmHg	2
	≤ 60 mmHg	3
PaCO ₂ (mmHg)* (breathing room air)	<40 mmHg	1
	40–45 mmHg	2
	>45 mmHg	3

The score of severity for each child was calculated by the sum of points obtained by each parameter evaluated; *Only for children showing very severe exacerbation.

Ethical issues

The ethical rules of the Declaration of Helsinki and those of the Brazilian National Council of Health, Ministry of Health, for experimentation with humans were strictly observed throughout this investigation. This work received approval from the Ethics Committee of the Faculty of Medicine, University of Brasilia (process number 038/1) and the Secretary of Health of Federal District (Foundation for Research and Education in Health Sciences) (process number 002/2002). All parents provided formal informed consent for their child's participation in this study.

Evaluation of peripheral blood eosinophil activation

The eosinophils were obtained by allowing them to adhere to microscope slides, which were marked with a 7 mm in diameter, round field using oil ink mixed with epoxy resin,

and using a technique adapted from Muniz-Junqueira et al. (14). Briefly, whole blood was collected without any anticoagulant, and 40 μ l was placed directly on each slide field. The slides were then incubated at 37°C for 45 min in a wet chamber to allow white blood cells (eosinophils, neutrophils, and monocytes) to adhere to the glass. The slides were then washed with a 0.15 M phosphate-buffered saline (PBS; pH 7.2) at 37°C to eliminate nonadhered cells, fixed with absolute methanol, and stained with a 10% Giemsa solution. The morphology of the eosinophils was assessed by evaluating 200 eosinophils by microscopy, in duplicate for each individual. Microscopic fields were randomly selected throughout the preparation. The slides were identified only at the end of the evaluation.

For each child, the grade of eosinophil activation was quantified by microscopy as the percentage of eosinophils showing one or more of the following morphological changes: single or multiple pseudopods; cytoplasmic vacuoles;

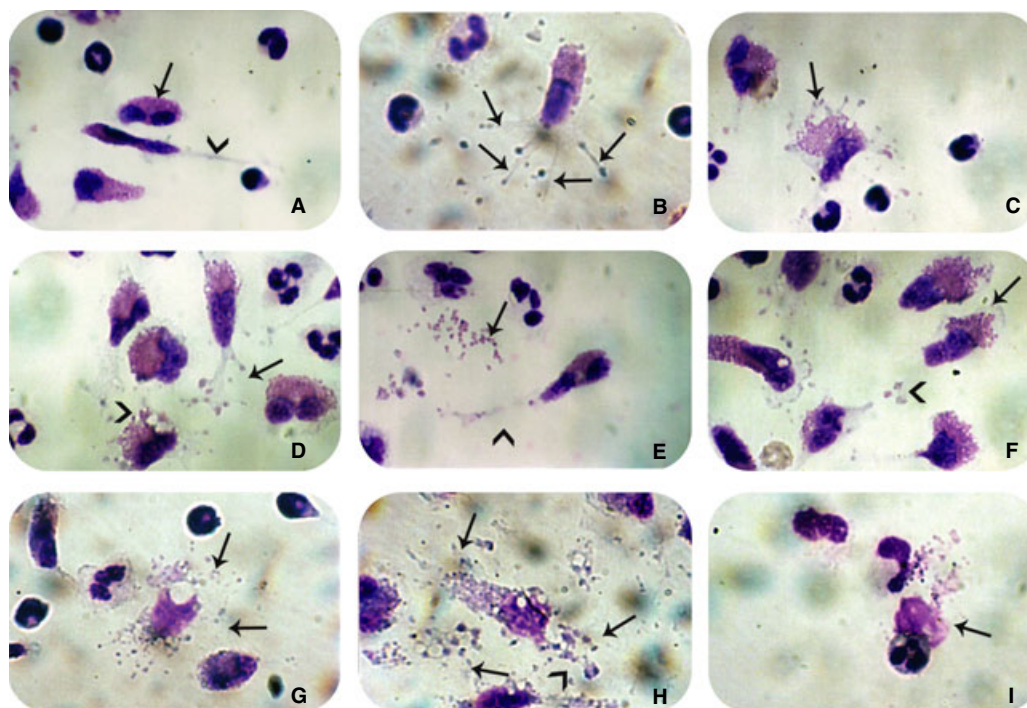


Figure 1 The figure illustrates some characteristic morphological changes in activated eosinophils (1000 $\times$ magnification, Olympus BH2 microscope). (A) A normal eosinophil (arrow) and an eosinophil emitting a single pseudopod (arrowhead). (B) The eosinophil is emitting multiple pseudopods (arrows), is spread, and is also releasing a small quantity of granules. (C) The eosinophil is spread, has vacuoles, and is emitting small quantity of granules (arrow). (D) The eosinophil is spread and releasing a small quantity of granules (arrow), and in the arrowhead, the eosinophil is spread, is releasing small quantity of granules, and is presenting vacuoles. (E) The eosinophil is emitting a single pseudopod and a small quantity of granules (arrowhead), and there is a cluster of free eosinophil granules (arrow). Observe that there is no eosinophil highly activated nearby the cluster of free eosinophil granules. (F) Arrowhead, the

eosinophil is releasing small quantity of granules and the two eosinophils in the arrow are spread and releasing a small quantity of granules. (G) The eosinophil is presenting vacuoles, is spread, and is emitting moderate quantity of granules. Observe that the eosinophil is emitting several granules below (arrowhead), in the right side and above (arrow) the cell. (H) The eosinophil is spread, has vacuoles, is emitting multiple pseudopods, and is releasing a large quantity of granules. Observe that there is emission of granules in large amount in all the edges of the eosinophil (arrow). Note that the contour of the cell (arrowhead) is less well defined, suggesting that the eosinophil is losing the membrane integrity. (I), a dead eosinophil is shown. Note the complete loss of plasma membrane integrity, there are rests of granules near the nucleus, and the nucleus is already showing signs of swelling.

a small, moderate or large granule release; and those with spreading, eosinophil death (apoptosis or necrosis), or a cluster of free eosinophil granules (15–17).

The sensitivity, specificity, positive predictive value, and negative predictive value of the eosinophil morphological changes in predicting the severity of an acute asthma exacerbation were calculated conventionally (18).

Statistical analysis

Analysis employed nonparametric statistics, and the results were expressed as medians and quartiles. The Mann–Whitney test was used to compare two unrelated samples. The Spearman's correlation coefficient was used to evaluate the relationship between the percentage of eosinophils with morphological changes and the severity score of the acute asthma exacerbation. A two-tailed $P < 0.05$ was considered significant. The Prism 5 (GraphPad Software, Inc., San Diego, CA, USA, 2005) was used for statistical analysis and graphics.

Results

Morphological aspects of activated eosinophils

Figure 1 illustrates the morphological aspects of activated eosinophils: normal eosinophil; eosinophils emitting single or multiple pseudopods; presence of cytoplasmic vacuoles; a small, moderate, or large granule release; spreading; a cluster of free eosinophil granules; and eosinophil death. Figure 1F, G, H illustrate small, moderate, and large quantity of granules release, respectively, with a gradual increase in granules around the eosinophils from F to H. Note that in F (small quantity), there are only a few granules in a localized area (arrow). In G (moderate quantity), the eosinophil is emitting several granules below (arrowhead), in the right side and above (arrow) the cell. In H (large quantity), a highly activated eosinophil is emitting large quantity of granules from all the sides of the cell (arrow); note that the contour of the eosinophil is less well defined (arrowhead). This suggests that the eosinophil is losing the plasma membrane integrity

caused by its highly activated state. In Fig. 1E, there is a cluster of free eosinophil granules (arrow). Note that there is no eosinophil highly activated nearby the cluster of free eosinophil granules. Figure 1I shows a dead eosinophil (arrow). Note that there is a complete loss of plasma membrane integrity, there are only rests of granules near the nucleus, and the nucleus is already showing signs of swelling. The aspect of the dead eosinophil was always as shown here, and this morphological appearance suggests that the death occurred by eosinophil cytolysis.

Eosinophils of asymptomatic asthmatic children were more activated than those of healthy children

The median percentages of activated eosinophils for each of the morphological changes were all higher in the asymptomatic asthmatic children than in the control group, as shown in Table 2 and Fig. 2. The median percentage of eosinophils with single pseudopod was twofold higher in asthmatic children than in the control group (10% vs 5%; $P < 0.0001$) (Fig. 2A). The median percentage of eosinophils with multiple pseudopods was 2% vs 0% for the control group ($P < 0.0001$) (Fig. 2B). Five percent of the eosinophils had cytoplasmic vacuoles in asthmatic children, whereas 0% had vacuoles in the control group ($P < 0.0001$) (Fig. 2C). The median percentage of eosinophils releasing a small (20% vs. 9%) ($P < 0.0001$), moderate (10% vs. 5%) ($P < 0.0001$), or large (11% vs. 4%) ($P < 0.0001$) quantity of granules was also significantly higher in asthmatic children than in the control group (Fig. 2D, E, F). Thirty percent of the eosinophils were spread in asthmatic children, whereas only 13% were spread in the control group ($P < 0.0001$) (Fig. 2H).

Eosinophils of children with an acute asthma exacerbation were more activated than those of asymptomatic asthmatic children

To verify whether an acute asthma exacerbation could produce an additional increase in eosinophil activation, the medians for each morphological change were compared

Table 2 Median (25–75th percentiles) of morphological changes in activated eosinophils in groups of children with asymptomatic asthma and acute asthma exacerbation and in the control healthy children

Eosinophil changes	Control ($n = 15$)	Asymptomatic asthma ($n = 15$)	$P^\dagger$	Acute asthma exacerbation ($n = 40$)	$P^\S$
Emitting single pseudopod	5 (4–6)	10 (9–11)	<0.0001	11 (9.2–16)	0.04
Emitting multiple pseudopods	0 (0–1)	2 (2–3)	<0.0001	7 (5–10)	<0.0001
Eosinophils having vacuoles	0 (0–2)	5 (4–6)	<0.0001	13.5 (11–20)	<0.0001
Releasing a small quantity of granules	9 (8–10)	20 (18–21)	<0.0001	28 (10–30)	0.40
Releasing a moderate quantity of granules	5 (4–6)	10 (9–11)	<0.0001	16 (13–29.7)	<0.0001
Releasing a large quantity of granules	4 (3–4)	11 (10–12)	<0.0001	7 (5–31.5)	0.43
Clusters of free eosinophil granules	1 (1–2)	5 (4–6)	<0.0001	10 (9–11)	<0.0001
Spread eosinophils	13 (11–15)	30 (28–31)	<0.0001	54.5 (49.2–70.7)	<0.0001
Eosinophil death	0 (0–1)	1 (1–2)	0.011	2 (1–3)	0.12

Comparison between the asymptomatic asthma vs control groups (†), and the acute asthma exacerbation vs asymptomatic asthma groups (§) used the Mann–Whitney test.

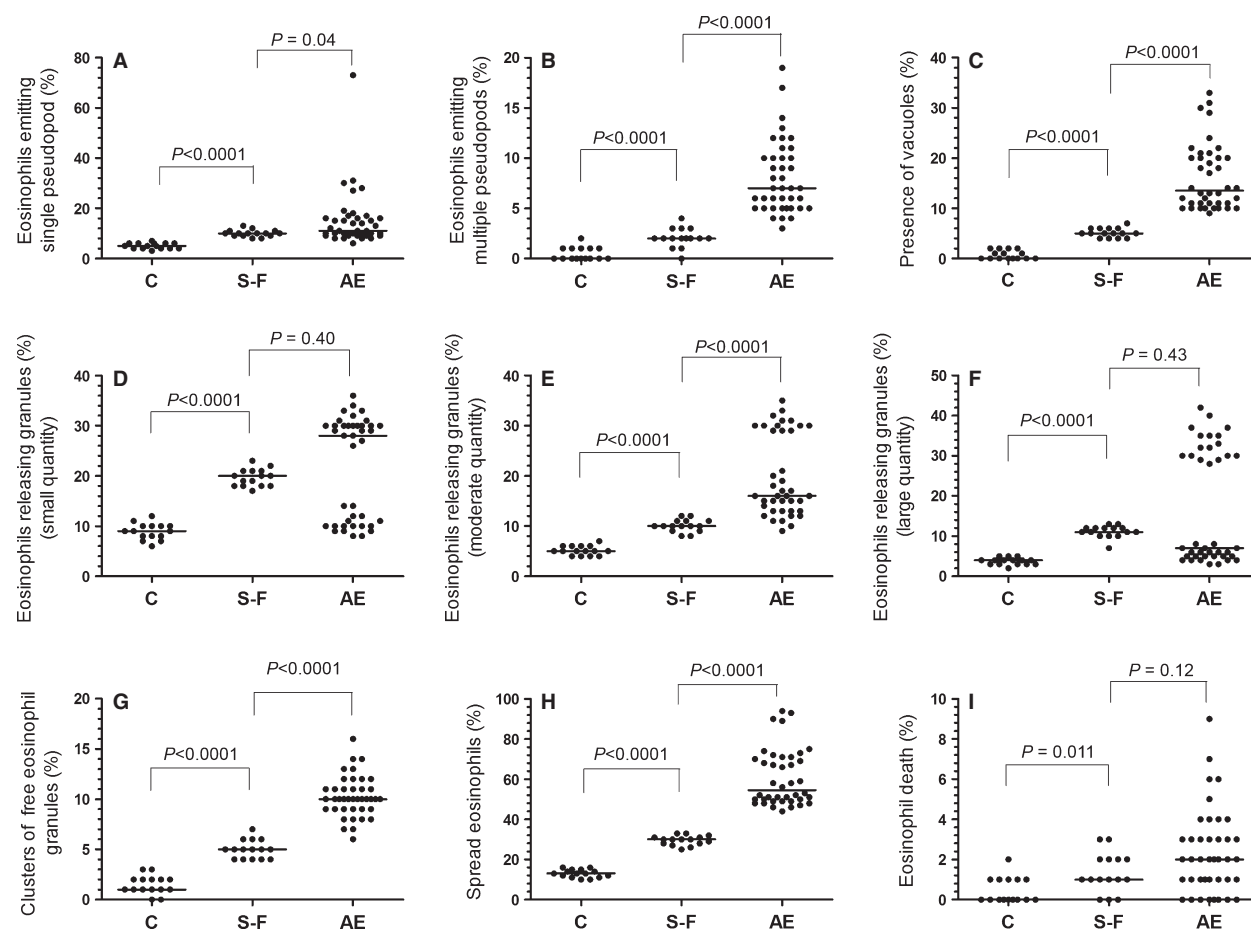


Figure 2 Percentage of activated eosinophils with morphological changes in children with acute asthma exacerbation (AE, $n = 40$) and symptom-free (S-F, $n = 15$) and in control healthy children (C, $n = 15$). All parameters of eosinophil activation were significantly increased in asthmatic children (Mann–Whitney test). Emission of

single and multiple pseudopods, having vacuoles, releasing of a moderate quantity of granules, spread eosinophils, and presence of clusters of free eosinophil granules were significantly increased in the group of children in acute asthma exacerbation (Mann–Whitney test).

between the groups of children with asymptomatic asthma and children with an asthma exacerbation. Table 2 and Fig. 2 show the results of the comparisons. Except for granule release in small and large quantities and eosinophil death, all the other morphological changes were higher in children with an acute asthma exacerbation than in the asymptomatic asthmatics.

The eosinophil morphological changes that were more significantly enhanced in an acute asthma exacerbation were multiple pseudopods, cytoplasmic vacuoles, the spreading of eosinophils, and clusters of free eosinophil granules (Fig. 2B, C, G, H).

Morphological changes in eosinophils correlate with the severity of an acute asthma exacerbation

An acute asthma exacerbation was classified in the following three levels of severity according to the clinical score values:

mild/moderate (score of 0–13), severe (score of 14–21), and very severe (score of ≥ 22).

To evaluate whether the eosinophil morphological changes could differentiate mild/moderate from severe asthma exacerbations, the medians of each morphological change were compared between these conditions (Table 3 and Fig. 3). The eosinophil changes were more frequent in a severe exacerbation than in mild/moderate one ($P < 0.0001$), except for the clusters of free eosinophil granules (Table 3). In addition, there was a significantly lower release of a small quantity of granules in the severe group compared with the mild/moderate group. All these more frequent morphological changes also showed correlation one another (Spearman, $P < 0.0001$).

Single and multiple pseudopods, cytoplasmic vacuoles, and the spread of eosinophils were also morphological changes that differentiated the very severe cases from the mild/moderate and severe asthma exacerbations (Fig. 3A, B, C, H).

Table 3 Median (25–75th percentiles) of morphological changes in activated eosinophils in groups of children with mild/moderate, severe, and very severe acute asthma exacerbation

Eosinophil changes	Mild/Moderate (<i>n</i> = 25)	Severe (<i>n</i> = 11)	<i>P</i> †	Very severe (<i>n</i> = 4)	<i>P</i> §	Percent of changes able to discriminate severity*
Emitting single pseudopod	10 (9–11)	16 (15–17)	<0.0001	29 (27.2–30.7)	0.004	≥ 14%
Emitting multiple pseudopodes	6 (5–7)	10 (9–11)	<0.0001	13.5 (12.2–16.2)	0.005	≥ 8%
Eosinophils having vacuoles	11 (10–13)	20 (18–22)	<0.0001	30.5 (29.2–32.5)	0.004	≥ 17%
Releasing a small quantity of granules	30 (28.5–31)	10 (9–11)	<0.0001	11 (9.2–13.5)	0.28	≤ 14%‡
Releasing a moderate quantity of granules	15 (12–16)	30 (30–32)	<0.0001	19.5 (18.2–20.7)	0.004	ND
Releasing a large quantity of granules	5 (4–7)	30 (29–33)	<0.0001	38.5 (35.5–41.5)	0.01	≥ 28%
Clusters of free eosinophil granules	10 (9–12)	10 (8–10)	0.17	10.5 (8.5–14.7)	0.34	ND
Spread Eosinophils	50 (48–52.5)	71 (68–73)	<0.0001	91.5 (89.2–93.7)	0.005	≥ 66%
Eosinophil death	1 (0–2)	3 (2–4)	0.0006	6.5 (6–8.5)	0.004	ND

ND, not discriminate.

Comparison between the mild/moderate vs severe groups (†), and the severe vs very severe groups (§) using the Mann–Whitney test.

*Percent change able to discriminate severity (mild/moderate vs severe + very severe). The minimum extreme values were considered as cutoffs for the respective sample distribution, except for releasing a small quantity of granules.

‡It was considered the maximum extreme value for the very severe group.

As shown in Fig. 3, there is a highly significant positive correlation between the exacerbation severity and each of the following eosinophil changes ($P < 0.0001$): single ($r_s = 0.70$) and multiple ($r_s = 0.76$) emission of pseudopods; presence of vacuoles ($r_s = 0.78$); releasing a moderate ($r_s = 0.80$) or large ($r_s = 0.77$) quantity of granules; and the spread of eosinophils ($r_s = 0.76$) and eosinophil death ($r_s = 0.60$). However, a significant negative correlation was noted for the emission of a small quantity of granules ($r_s = -0.75$; $P < 0.0001$), and no correlation was detected for clusters of free eosinophil granules ($P = 0.59$).

Considering the extreme values as cutoffs for the respective sample distribution, the minimum percentages of eosinophils with morphological changes that indicated a risk of a severe acute exacerbation were ≥ 14% for those emitting single pseudopod and 8% for multiple pseudopods, 17% for cytoplasmic vacuoles, 28% eosinophils releasing a large quantity of granules, and 66% for spread eosinophils (Fig. 3 and Table 3).

The sensitivity, specificity, positive predictive values, and negative predictive values for the eosinophil morphological changes in the asthma exacerbation group were calculated considering the mild/moderate cases vs the sum of the severe and very severe episodes. The lower extreme values from the severe group were considered for the calculations. As shown in Table 4, the most sensitive and specific morphological change was the spread of the eosinophils (100% and 96%, respectively), followed by the presence of cytoplasmic vacuoles, single pseudopod, and release of a large quantity of granules (100% and 92%, respectively).

Discussion

This study has clearly shown that morphological changes in peripheral blood eosinophils are more frequent in asthmatic children than in healthy children. Moreover, eosinophil

spreading, clusters of free eosinophil granules, the presence of vacuoles, and emission of multiple pseudopods were more frequently observed in children with an asthma exacerbation.

Another important finding was the positive correlation between the clinical severity level of the acute asthma exacerbation and the percent of activated eosinophils with morphological alterations. The emission of pseudopods, presence of vacuoles, eosinophil spreading, and emission of granules in high amounts were the changes that showed positive correlations. Furthermore, the emission of single and multiple pseudopods, presence of eosinophils presenting cytoplasmic vacuoles, and spreading of eosinophils may differentiate between mild/moderate, severe, and very severe forms of an acute exacerbation. These changes showed more sensitivity and specificity in differentiating between mild/moderate and severe forms of an acute exacerbation. Additionally, these changes are those that indicate a high eosinophil activation. In fact, these morphological changes showed high correlation one another, because they were expressing the same phenomenon of activation of the eosinophils. But several morphological changes were also individually able to discriminate the severity of an asthma exacerbation.

Functional properties of eosinophils can be divided into at least three stages: resting, partial activation, and full activation depending on stimuli (19). Conditions under which eosinophil activation occurs may generally exhibit a highly complex molecular milieu, to which both plasma-derived proteins and cell-derived mediators contribute (19, 20). Eosinophil granules are composed mainly of cytotoxic cationic proteins and store a plethora of cytokines, chemokines, and growth factors, available for very rapid, stimulus-dependent release. Eosinophils may release granules by a regulated piecemeal degranulation, by classical exocytosis, in which granules fuse with the plasma membrane to release their contents, and by cytolysis, in which the plasma membrane ruptures and granules are liberated extracellularly (20, 21).

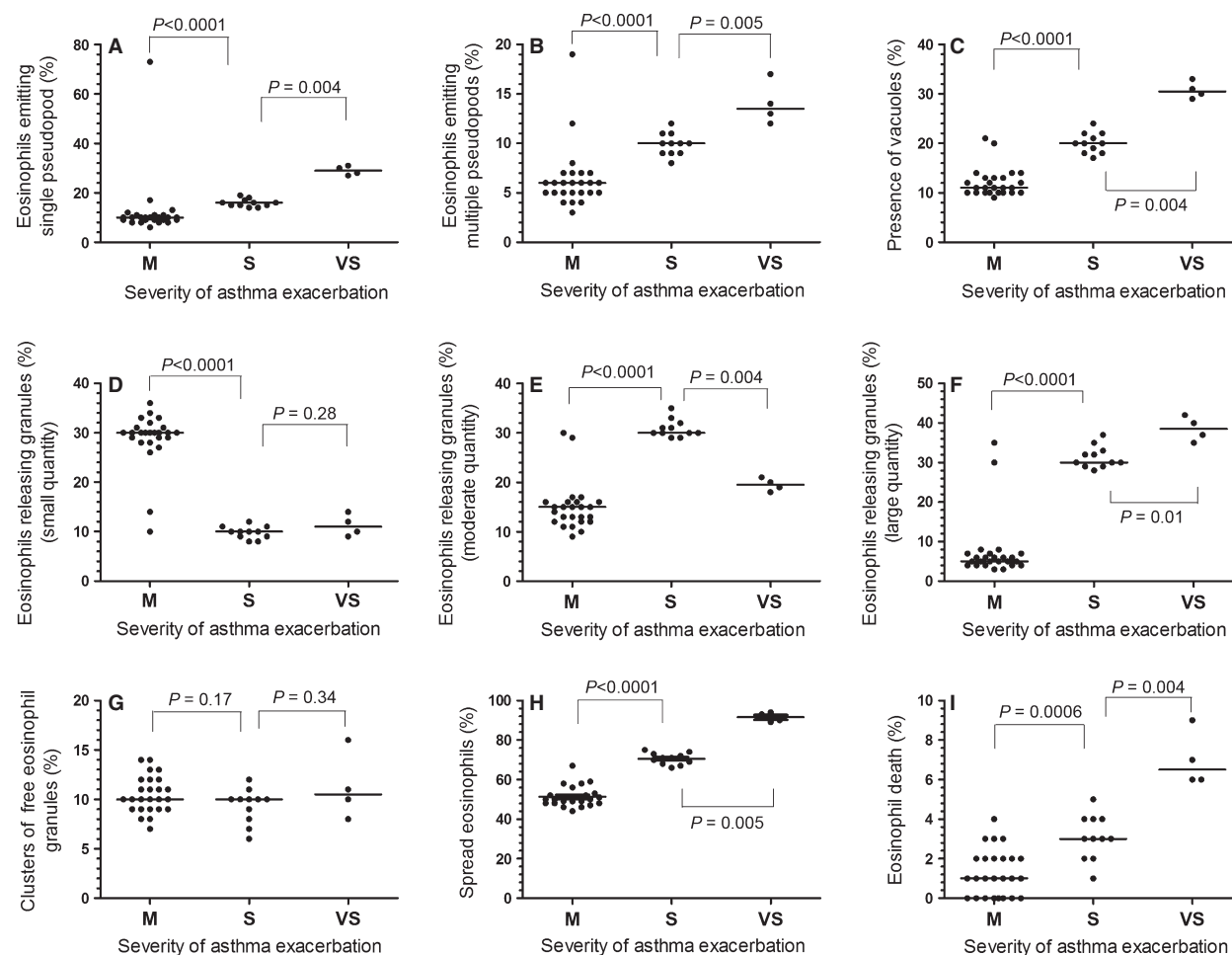


Figure 3 Percentage of activated eosinophils with morphological changes in 40 asthmatic children in acute exacerbation according to the clinical status characterized as mild/moderate (M) (score 0–13; $n = 25$), severe (S) (score 14–21; $n = 11$), and very severe (VS) (score ≥ 22 ; $n = 4$). The Mann–Whitney test was used for the

comparisons indicated. The correlation between the percentage of changed eosinophils and the severity of asthma exacerbation can also be appreciated and was tested by the Spearman' correlation (the coefficients of correlation and the respective P values are described in the text).

Activated eosinophils are effector cells with proinflammatory and destructive capabilities. Eosinophils with activation phenotypes are observed in biological specimens from patients with disease, and deposition of eosinophil products is readily seen in the affected tissues from these patients (19). Eosinophils abounding and degranulating in airway mucosal and pulmonary tissues are also believed to have pathogenic roles in asthma (20). In eosinophil degranulation by cytolysis, there are loss of plasma membrane integrity and release of toxic granules, and considerable evidence exists to link eosinophil granules proteins and severity of the disease (19). Observations of diseased airway mucosa suggest that toxic granules released by eosinophil cytolysis may damage mucosal cells (20) and the highly activated eosinophil also die in this process (19, 20). The poor discrimination of the contour of highly activated eosinophils releasing high quantity of granules presently observed (Fig. 1H) may be explained because possibly these cells are losing the plasma membrane

integrity and possibly are in death process (19, 21). The Fig. 1I shows a possible final evolution in this process, with the complete loss of plasma membrane integrity and finishing in the death of the eosinophil. These facts may explain the positive correlation between the clinical severity level of an acute asthma exacerbation and the grade of eosinophil activation represented by the percent of activated eosinophils, as presently observed. The morphological appearance of the dead eosinophil observed in the Fig. 1I suggests that death occurred by cytolysis (20). The activation of the eosinophils was possibly caused by the deregulation of immune system and the exacerbated cytokines production in severe asthma.

Berkart, as early as in 1880, depicted activated eosinophils in fresh sputum from an asthma patient, and Schmitz in 1892 observed eosinophils and free eosinophil granules in sputum samples, as cited by Persson and Erjefält (17). In peripheral blood, hypodense eosinophils have been consistently described in asthmatic individuals and represent acti-

Table 4 Sensitivity, specificity, positive predictive value, and negative predictive value of eosinophil morphological changes in the group of children with acute asthma exacerbation, according to mild/moderate vs severe plus very severe episodes

Eosinophil changes	Sensitivity, %	Specificity, %	Predictive value of positive result, %	Predictive value of negative result, %
Emitting single pseudopod	100	92	88.23	100
Emitting multiple pseudopods	100	88	83.33	100
Eosinophils having vacuoles	100	92	88.23	100
Releasing a small quantity of granules	93.3	96	93.3	96
Releasing a moderate quantity of granules	73.3	92	84.6	85.18
Releasing a large quantity of granules	100	92	88.23	100
Spread eosinophils	100	96	93.75	100
Eosinophil death	100	32	46.87	100

Sensitivity = (number of true positives/number of true positives + number of false negatives) × 100.

Specificity = (number of true negatives/number of true negatives + number of false positives) × 100.

Predictive value of positive result = (number of true positives/number of true positives + number of false positives) × 100.

Predictive value of negative result = (number of true negatives/number of true negatives + number of false negatives) × 100.

vated cells (16). The changes in light-density eosinophils include the appearance of cytoplasmic vacuoles, an alteration of the cell size and shape, an increase in the granule size, and these cells may be degranulated, contain less granules, have an increased cell volume, and upregulate adhesion molecules (16, 22). Our observations of the eosinophil morphology after adhering to the slide agree with these activated cell descriptions.

The morphological aspects of eosinophil activation have been evaluated using light (5), electronic (20, 23), and confocal microscopy (24). Pronk-Admiraal and colleagues also used light microscopy to study the morphological features that reflect the eosinophil's state of activation (5). The authors assessed the number of nuclear lobes, number of vacuoles, and density of granulation and concluded that the size of the blood eosinophils is a useful parameter to distinguish between patients with and without eosinophilia (5).

The methodological aspect in our study is important and should be emphasized. A method that permitted the eosinophils to adhere to the glass before evaluating of their activation was used. Our data suggest that the glass adherence may reveal the state of activation-associated morphology of these peripheral blood cells. This methodology appears to be a more reliable indication of what will happen when these cells migrate to the pulmonary tissue. It is possible that eosinophils that are more activated will have more degranulation in the target tissue, resulting in more severe clinical manifestations for the child. The correlation between the severity of asthma exacerbation and morphological changes observed in this work demonstrates the level of activation of eosinophil in these individuals. Using this technique to separate the polymorphonuclear cells directly from the whole blood has also been used to assess phagocytosis (14, 25, 26) and the influence of drugs on phagocyte functions (27, 28).

Enhanced eosinophil responses have critical roles in the development of asthma. IL-5 regulates the maturation, migration, and survival of eosinophils, and IL-5 and eotaxins mediate the trafficking and activation of eosinophils in inflamed tissues. *In vitro* eosinophil stimulation with eotaxin-

2 or PAF led to a spread morphology in a proportion of eosinophils, observed by confocal microscopy (24), and the eotaxin concentrations in the induced sputum supernatants were increased in subjects with severe asthma (29). The levels of these molecules are increased in asthmatic children (29) and may have induced the morphological changes in the eosinophils seen in the present work.

Morbidity and mortality in acute exacerbations of asthma are most often associated with an underassessment of the condition's severity, inadequate action at the onset of the exacerbation, and under-treatment of the exacerbations. Objective diagnostic measures are more reliable indicators of the severity than the degree of symptoms (13). Therefore, more objective measures of risk could help physicians diagnose and choose treatment for acute asthma exacerbations, which may rapidly improve the patient's clinical status.

Underestimating the clinical severity of the asthma exacerbation may cause physicians to misjudge the proper management of and therapy for patients, leading to an inadequate treatment and an increase in the morbidity and mortality. Supplementing the asthma assessment and management guidelines with simple laboratory techniques may improve the management of patients (30). The correlation of the severity of the asthma exacerbation with the morphological changes of activated eosinophils observed in our work suggests that the test described could be a simple and sensitive marker of clinical severity and help physicians adequately manage the disease.

Although the evaluation of morphological changes in eosinophils may have a potential clinical utility in diagnosis of the severity of an acute asthma exacerbation, prospective studies are required to confirm this utility. Perhaps a limitation on the utility of this test is their relative dependence on the technician's experience and skills to identify the eosinophil changes.

During recent years, there has been a growing interest in using noninvasive biomarkers to understand and monitor airway inflammation in subjects with respiratory tract disorders, mainly asthma (31, 32). Fleming et al. (33). showed that the

sputum eosinophil count was not able to guide management in children with severe asthma. It should be evaluated whether the morphological changes in eosinophils induced by treatment may have some value to following up therapy in asthmatic individuals.

In conclusion, our data showed that the morphological changes in eosinophils are an important consideration for the assessment of asthmatic children. These eosinophil changes markedly correlate with the disease and may indicate the clinical severity of the acute exacerbation. The emission of single and multiple pseudopods, cytoplasmic vacuoles, eosinophils releasing a large quantity of granules, and the eosinophil spreading may differentiate individuals with a mild/moderate exacerbation from those with a severe exacerbation. The practical and simple laboratory evaluation of eosinophils, as presently employed, requires only a minimal amount of peripheral blood and may help identify the severity of an asthma exacerbation, provide guidance for a proper clinical evaluation, and help manage acute asthma exacerbations in children.

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Author contributions

All authors participated in the conception and design of the study, analysis and interpretation of data, elaboration of the manuscript, and have approved the content of it. SMB-M had a major contribution to recruitment of volunteers and performing human experiments. LFJJ had a major contribution to analysis and interpretation of data and preparing and reviewing the manuscript. MIM-J coordinated this investigation, contributed in the conception, design of the study, analysis and interpretation of data, and preparing and reviewing the manuscript.

Conflict of interest

None of the authors has any conflict of interest.

References

- GINA, Global strategy for asthma management and prevention 2010 [2012 February 13]. Available from <http://www.ginasthma.org>.
- Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;**360**:1715–1721.
- Löwhagen O. Diagnosis of asthma – a new approach. *Allergy* 2012;**67**:713–717.
- Fahy JV. Eosinophilic and neutrophilic inflammation in asthma. Insights from clinical studies. *Proc Am Thorac Soc* 2009;**6**:256–259.
- Pronk-Admiraal CJ, van Haaster FE, Bartels PC. Clinical evaluation of changes in the morphology of eosinophils. *Clin Chem Lab Med* 2002;**40**:27–31.
- Adamko DJ, Odemuyiwa SO, Vethanayagam D, Moqbel R. The rise of the phoenix: the expanding role of the eosinophil in health and disease. *Allergy* 2005;**60**:13–22.
- Hogan SP. Recent advances in eosinophil biology. *Int Arch Allergy Immunol* 2007;**143**:S3–S14.
- Wardlaw AJ, Brightling C, Green R, Woltmann G, Pavord I. Eosinophils in asthma and other allergic diseases. *Br Med Bull* 2000;**56**:985–1003.
- Jatakanon A, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. *Am J Respir Crit Care Med* 2000;**161**:64–72.
- Pizzichini E, Pizzichini MM, Efthimiadis A, Dolovich J, Hargreave FE. Measuring airway inflammation in asthma: eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood. *J Allergy Clin Immunol* 1997;**99**:539–544.
- Gogate S, Katial R. Pediatric biomarkers in asthma: exhaled nitric oxide, sputum eosinophils and leukotriene E4. *Curr Opin Allergy Clin Immunol* 2008;**8**:154–157.
- Fritscher CC, Solé D, Rosário N, Fiterman J, Pastorino AC, Pereira LFF et al. III Consenso Brasileiro no Manejo da Asma. *J. Pneumol* 2002; **28**: S1–S28.
- International consensus report on diagnosis and management of asthma. *National Heart, Lung, and Blood Institute*. National Institute of Health, Bethesda Maryland. NIH Publication 92-3091, 1992: 1–72.
- Muniz-Junqueira MI, Peçanha LMF, Silva-Filho VL, Cardoso MCA, Tosta CE. Novel microtechnique for assessment of postnatal maturation of the phagocytic function of neutrophils and monocytes. *Clin Diagn Lab Immunol* 2003;**10**:1096–1102.
- Gleich GJ, Adolphson CR, Leiferman KM. The biology of the eosinophilic leukocyte. *Annu Rev Med* 1993;**44**:85–101.
- Kroegel C, Virchow JC Jr, Luttmann W, Walker C, Warner JA. Pulmonary immune cells in health and disease: the eosinophil leukocyte (Part I). *Eur Respir J* 1994;**7**: 519–543.
- Persson CG, Erjefält JS. “Ultimate activation” of eosinophils in vivo: lysis and release of clusters of free eosinophil granules (Cfegs). *Thorax* 1997;**52**:569–574.
- Keren DF, Hrusczyk VA. Clinical significance and cost-effectiveness of clinical immunology testing. In: Rose NR, de Macario EC, Folds JD, Lane HC, Nakamura RM, editors. *Manual of Clinical Laboratory Immunology*. Washington: American Society for Microbiology Press, 1997: 1206–1212.
- Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev* 2011;**242**:161–177.
- Erjefält JS, Persson CGA. New aspects of degranulation and fates of airway mucosal eosinophils. *Am J Respir Crit Care Med* 2000;**161**:2074–2085.
- Shamri R, Xenakis JJ, Spencer LA. Eosinophils in innate immunity: an evolving story. *Cell Tissue Res* 2011;**343**:57–83.
- Neves JS, Weller PF. Functional extracellular eosinophil granules: novel implications in eosinophil immunobiology. *Curr Opin Immunol* 2009;**21**:694–699.
- Melo RC, Weller PF, Dvorak AM. Activated human eosinophils. *Int Arch Allergy Immunol* 2005;**138**:347–349.
- Lacy P, Willets L, Kim JD, Lo AN, Lam B, MacLean EI et al. Agonist activation of F-actin-mediated eosinophil shape change and mediator release is dependent on Rac2.

- Int Arch Allergy Immunol* 2011;**156**:137–147.
25. Muniz-Junqueira MI, Mota LM, Aires RB, Junqueira LF Jr. Differing phagocytic function of monocytes and neutrophils in Chagas' cardiopathy according to the presence or absence of congestive heart failure. *Rev Soc Bras Med Trop* 2004;**37**:447–453.
26. Muniz-Junqueira MI, Braga Lopes C, Magalhães CA, Schleicher CC, Veiga JP. Acute and chronic influence of hemodialysis according to the membrane used on phagocytic function of neutrophils and monocytes and pro-inflammatory cytokines production in chronic renal failure patients. *Life Sci* 2005;**77**:3141–3155.
27. Muniz-Junqueira MI, Mota LM, Aires RB, Junqueira LF Jr. Digitalis inhibits and furosemide does not change the in vitro phagocytic function of neutrophils of healthy subjects. *Int Immunopharmacol* 2003;**3**:1439–1445.
28. Muniz-Junqueira MI, de Paula-Coelho VN. Meglumine antimonate directly increases phagocytosis, superoxide anion and TNF-alpha production, but only via TNF-alpha it indirectly increases nitric oxide production by phagocytes of healthy individuals, *in vitro*. *Int Immunopharmacol* 2008;**8**:1633–1638.
29. Zietkowski Z, Tomasiak-Lozowska MM, Skiepkowski R, Zietkowska E, Bodzenta-Lukaszyk A. Eotaxin-1 in exhaled breath condensate of stable and unstable asthma patients. *Respir Res* 2010;**11**:110.
30. Koshak EA, Alamoudi OS. Do eosinophil counts correlate differently with asthma severity by symptoms versus peak flow rate? *Ann Allergy Asthma Immunol* 1999;**83**:567–571.
31. Bakakos P, Schleich F, Alchanatis M, Louis R. Induced sputum in asthma: from bench to bedside. *Curr Med Chem* 2011;**18**:1415–1422.
32. van der Valk RJP, Baraldi E, Stern G, Frey U, de Jongste JC. Daily exhaled nitric oxide measurements and asthma exacerbations in children. *Allergy* 2012;**67**:265–271.
33. Fleming L, Wilson N, Regamey N, Bush A. Use of sputum eosinophil counts to guide management in children with severe asthma. *Thorax* 2012;**67**:193–198.