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Hydrogen Peroxide in Exhaled Breath Condensate in Asthmatic Children during Acute Exacerbation and after Treatment

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Key Words
Asthma exacerbation · Biomarkers · Hydrogen peroxide · Children · Inflammation and oxidative stress · Paediatric allergy

Abstract
Background: In asthmatics, the concentration of hydrogen peroxide (H_2O_2) in exhaled breath condensate (EBC) has been found to be increased and to be related to airway inflammation. ***Objective:*** The aim of this study was to determine whether in children with acute exacerbation, exhaled H_2O_2 levels could be influenced by treatment and linked to airway obstruction. ***Methods:*** Twenty-two asthmatic children (mean age 9.4 years, range 6–14) with asthma exacerbation and 12 healthy children (mean age 11.7 years, range 7–15) were enrolled. Concentrations of exhaled H_2O_2 before and after standard treatment for asthma attack were compared with those of controls and with clinical observation. Asthmatic children and controls underwent spirometry and skin prick tests to common aeroallergens. ***Results:*** Exhaled H_2O_2 concentrations were significantly higher in children with asthma both before (median 0.273 μM; $p < 0.001$) and after pharmacologic treatment (median 0.303 μM; $p = 0.001$) compared to control values (median 0.045 μM). After treatment, exhaled H_2O_2 concentrations remained significantly higher in children with and without auscultatory wheezing than in controls ($p = 0.034$ and $p < 0.001$, respectively). EBC H_2O_2 levels in asthmatics before treatment did not differ from those after treatment. No correlation was found between H_2O_2 and forced expiratory volume in 1 s values. All asthmatics but one were atopics. ***Conclusions:*** In children with acute asthma exacerbation, exhaled H_2O_2 concentrations in EBC are significantly elevated. In the short-term follow-up, H_2O_2 levels remain at high levels and are not correlated with lung function or improvement in symptoms.

Introduction

Exhaled breath condensate (EBC), which is a fluid collected by cooling of exhaled air during tidal breathing, is a completely non-invasive method, easy to perform and applicable to children [1]. EBC contains a large number of molecules originating from the airways, which are ex-

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pired as bioaerosol [2, 3], whose concentration cannot entirely be compared directly to information derived from bronchoalveolar lavage [4]. Despite methodological problems, such as lack of reference values of biomarkers from healthy subjects, full standardization of the procedure or the sensitivity of the available assays [5], markers in EBC have been proposed as a suitable method for the assessment of airway inflammation in asthmatic patients [6–9]. Increased oxidative stress, defined as an imbalance between oxidants and antioxidants, is involved in airway inflammatory diseases including asthma [10]. Activated inflammatory cells, especially eosinophils but also macrophages and neutrophils, generate several reactive oxygen species which consequently increase gene expression of inflammatory mediators, damage epithelial cells and increase bronchial hyperreactivity [11]. Superoxide anion (O_2^-) is rapidly metabolized by superoxide dismutase to form hydrogen peroxide (H_2O_2) which in the airways tends to evaporate in the exhaled air, and therefore, H_2O_2 in EBC may be considered a marker of oxidative stress. However, H_2O_2 is generated from a multiplicity of sources including, but not limited to, inflammatory cells and has been associated with different lung diseases [12]. Therefore, as a diagnostic means, H_2O_2 may lack specificity. Previous studies on exhaled H_2O_2 levels were performed mainly in clinically stable asthmatics, and authors reported that EBC H_2O_2 values were generally high [13–15] and related to the number of eosinophils in sputum as well as to airway hyperresponsiveness intensity [16].

The treatment of children with acute asthma is based on asthma severity as assessed by clinical signs and symptoms, measurement of pulse oximetry, pulmonary function and blood gases [17]. These parameters do not take into consideration the degree of oxidative stress or inflammation in the airways. In our study, we have investigated EBC H_2O_2 levels in children with acute asthma exacerbation before and after a 7-day treatment. In addition, we determined the relationship between exhaled H_2O_2 concentrations and lung function parameters.

Methods

Subjects

We conducted a study in children aged 6–14 years with physician-diagnosed asthma, whose cases were followed at two hospital-based out-patient asthma clinics. During periodical visits that were scheduled in the morning, children who had an acute episode of asthma were consecutively enrolled in this longitudinal study between February and June 2008. Children admitted to the study had a history of physician-diagnosed asthma according to international criteria [17]. Briefly, diagnosis of asthma was based on recurrent symptoms such as breathlessness, wheezing, cough and chest tightness and was confirmed through follow-up, observing the response to a bronchodilator and to anti-inflammatory treatment [17]. Children were required to have had at least three previous episodes of wheezing treated with inhaled bronchodilators. Reversibility of airflow limitation after short-acting β_2-agonist administration was also measured by spirometry [17]. Children were required to have a history of asthma exacerbations triggered by an acute upper respiratory tract infection or allergens. An acute upper respiratory infection was defined by a history of acute onset of rhinitis and/or otitis and/or sore throat with or without fever accompanied by erythema and/or mucosal swelling and/or purulent secretion.

Children with a history of intermittent or mild-moderate persistent asthma were admitted to this study whether they were on a pharmacologic long-term treatment with inhaled steroids, long-acting β_2-agonists or anti-leukotrienes or not.

We excluded all subjects who met the following criteria: severe asthma attack requiring hospitalization, bronchial provocation test in the last week, chronic upper respiratory infection, chronic cardiopulmonary disease, concurrent pneumonia, nasal polyps, obesity, gastro-oesophageal reflux and aspirin-induced asthma. Aspirin-induced asthma was excluded by a history of no temporal relationship between aspirin intake and asthma symptoms. Gastro-oesophageal reflux was excluded on the basis of clinical history and physical examination [18].

Acute exacerbation was defined by evidence of wheeze, dyspnoea, tachypnoea and/or use of accessory respiratory muscle, with/without desaturation in a child with a history of asthma, and no clinical evidence of lower respiratory tract infection (fever, focal crepitations, pleuritic pain) [19]. Children who had taken systemic steroids in the last 3 weeks were excluded from the study.

A group of healthy age-matched children without asthma and atopic diseases was enrolled as control group. Other inclusion criteria were no intake of corticosteroids in the last 3 weeks and no respiratory tract infections in the last 4 weeks. Skin prick test (SPT) results to aeroallergens in the control group were negative.

Study Design

At recruitment (visit 1), children underwent physical examination. Oxygen saturation, heart rate and respiratory rate were recorded. Details of current medication were requested. SPTs were performed. Asthmatic children were treated according to international guidelines [17] after collection of an EBC sample for H_2O_2 detection and lung function measurement. Briefly, 2–4 puffs of short-acting β_2-agonists were given every 20 min to 4 h in relation to the severity of the symptoms. The 4-hourly administration was continued for 1 week. In case of a poor immediate response or a moderate-severe exacerbation [17], inhaled ipratropium bromide and oral corticosteroids (prednisolone 1 mg/kg/day) were given for 7 days. Oxygen was administered if oxygen saturation was <95%. During the treatment period, the parents filled in a clinical diary of coughing and wheezing (score: 0 = none, 1 = mild, 2 = severe), as well as of drug administration. Parents were instructed to mark wheezing when the child had difficult breathing and/or musical noise in the chest like a whistle. One point was assigned to each type of drug administered per day. After 1 week of treatment, children underwent a second visit and

performed collection of EBC and pulmonary function. The treatment was considered effective if a child had no symptom or sign of asthma and oxygen saturation was >95%.

A control group of healthy children underwent EBC collection, SPT and measurement of lung function. All the study procedures were conducted in the morning. Each visit was performed independently by two physicians. In the case of disagreement, a third physician was consulted. The University Ethical Committee of Parma approved the protocol and all parents gave their informed consent.

Methods

EBC was collected and processed according to the American Thoracic Society/European Respiratory Society recommendations [2], using commercial condensers. Ecoscreen (Jaeger, Hochberg, Germany) with a single-exit valve, in order to separate the expiratory flow from the inspiratory flow, was available for children recruited in Milan. Turbo-Deccs (Medivac, Parma, Italy) with a one-way valve and a reliable saliva trap, connected to a collecting vial (50 ml) by means of a tube [20, 21], was available for children enrolled in Parma. Each subject used the same type of condenser for both visits. The collecting temperature in Turbo-Deccs was –5°C, and in Ecoscreen, it is reported to be from –10 to –20°C. To each patient a nose clip was applied to exclude possible contamination of nasal origin. The children breathed tidally through the mouth for 15 min or for a volume equal to 2 ml, while sitting comfortably. They kept their mouth dry during EBC collection by periodically swallowing excess saliva. The collection was stopped in case of cough or excessive saliva and was restarted when the episode has resolved. Each collection was performed 10 min after the last forced expiratory manoeuvre. For each patient, approximately 2 ml of EBC was collected in cooling vials. The collected EBC samples were stored at –80°C in polypropylene tubes until analysed. Samples were analysed no later than 1 month after the collection. Hydrogen peroxide was measured as previously described [22, 23], spectrofluorometrically using commercial kit Amplex Red Hydrogen Peroxide (Molecular Probes, Eugene, Oreg., USA). Salivary contamination was measured by means of the colorimetric detection of α-amylase (Infinity Amylase Reagent, Sigma, Milan, Italy).

Lung function measurement was performed according to the European Respiratory Society/American Thoracic Society guidelines [24] using an electronic spirometer (Masterscope; Jaeger, Wuerzburg, Germany). Forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), the FEV_1/FVC ratio, and forced expiratory flow 25–75% ($FEF_{25–75}$) were expressed as percentage of predicted reference values. Patients did not receive inhaled bronchodilators until at least 4 h before EBC collection and spirometry.

Skin prick testing was performed with a panel of standardized common allergen extracts: birch, hazel, grasses, mugwort, alternaria, cat epithelium, dog epithelium, pellitory and house dust mite. A positive SPT was defined as a wheal with a mean diameter of at least 3 mm greater than the saline control after 15 min [25].

Statistics

The distribution of the variables was assessed by the Kolmogorov-Smirnov test; normally distributed variables are presented as the mean ± SD, and non-normally distributed variables as the median and interquartile range (IQR). Differences between continuous variables were calculated by the Mann-Whitney test, the Wilcoxon signed rank test, the two-tailed Student t test or ANOVA when appropriate. The χ^2 test or Fischer's exact test were used to compare ordinary variables. Correlations were expressed as Spearman's correlation coefficient. A p value <0.05 was considered statistically significant. A study group of at least 20 subjects and a control group of at least 10 subjects were determined to detect 1.25 SD difference at the 5% significance level with power 90%.

Table 1. Characteristics of the 22 asthmatic children

Characteristics	Asthmatic children
Mean age ± SD, years	9.4 ± 2.70
Males/females	15/7
Positive SPT	
Grasses	16 (72)
House dust mites	12 (54)
Alternaria alternata	8 (36)
Dog epithelium	8 (36)
Cat epithelium	8 (36)
Ambrosia	5 (22)
Hazel	3 (1.3)
Birch	3 (1.3)
Aspergillus fumigatus	3 (1.3)
Pellitory	2 (0.9)
Plantago lanceolata	2 (0.9)
At least one positive SPT	21 (95)
Monosensitized patients	9 (41)
Polysensitized patients	13 (59)
Long-term medications	
Salmeterol+fluticasone dipropionate	7
Fluticasone dipropionate	2
Montelukast	1

Figures in parentheses are percentages.

Results

Twenty-two asthmatic children with acute asthma exacerbation were consecutively enrolled. Demographic and clinical characteristics are shown in table 1. On the basis of medical history, physical examination and concordant SPT results, exacerbation was presumed to be due to seasonal allergen exposure in 16 cases and to upper respiratory tract infection in 6 cases. At visit 1, all children had wheezing documented by auscultatory wheeze, cough, shortness of breath and/or chest tightness and/or use of accessory respiratory muscle. At the second visit, 13 (61%) of the 22 patients were asymptomatic with a normal chest auscultation. In the remaining 9 children, auscultatory wheeze was still present, without using accessory breathing muscles or dyspnoea. During the 7-day

Table 2. Exhaled H_2O_2 concentrations (μM) in controls and asthmatic children

	Asthmatic children (n = 22)	Control subjects (n = 12)	p value
Before treatment	0.273, 0.142–0.669	0.045, 0.017–0.082	0.001
After treatment	0.303, 0.122–0.723		0.001

Data are presented as medians and IQRs.

Table 3. H_2O_2 values (μM) in children who were on long-term treatment with inhaled glucocorticosteroids and in controls

	Before treatment	After treatment
Control subjects (n = 12)	0.045, 0.017–0.082[a, b, c, f]	
Inhaled glucocorticosteroids		
Yes (n = 9)	0.25, 0.16–0.49[a, d]	0.24, 0.14–0.56[e, f]
No (n = 13)	0.3, 0.14–0.81[b, d]	0.38, 0.12–0.72[e, c]

Data are presented as medians and IQRs.
[d, e] $p > 0.05$; [a, f] $p = 0.002$; [b] $p = 0.009$; [c] $p = 0.001$.

treatment, all patients received salbutamol, 12 ipratropium bromide and 5 oral steroids. In asthmatics, the mean symptom-drug score at day 1 was higher than that at day 7 (3.59 ± 1.3 vs. 2.59 ± 1.18; p = 0.005); this significant difference was also observed for symptoms alone (1.82 ± 1.01 vs. 1.05 ± 0.95; p = 0.006). At visit 2, the symptom-drug score was higher in children with wheezing than in those free of symptoms (p = 0.032). The maximum symptom score was 4 and the maximum symptom-drug score was 7. At the first visit, oxygen saturation was 96.9 ± 0.014% (range 94–98) and at the second visit 97.6 ± 0.007% (range 96–99; p = 0.726). The control group consisted of 12 healthy children (7 males, 5 females, with a mean age of 11.7 ± 2.75 years, range 7–15).

H_2O_2 Concentrations

All patients and controls performed a correct manoeuvre for the collection of EBC, producing a sufficient quantity for the analysis of H_2O_2. Salivary contamination was not detected in the EBC samples. At baseline and at visit 2, in children with asthma EBC, H_2O_2 values were significantly higher than those detected in healthy control children (table 2). In asthmatics, there was no significant difference in EBC H_2O_2 concentrations between visit 1 and visit 2 (p = 0.682). There was no statistically significant difference in EBC H_2O_2 values between the two condensers used. At baseline, values of EBC H_2O_2 obtained with Turbo-Deccs (n = 12, 0.195 μM, IQR 0.144–0.379) were similar to those with Ecoscreen (n = 10, 0.625 μM, IQR 0.158–0.912; p = 0.15) and higher than those of controls (p = 0.007 and 0.001, respectively). At visit 2, H_2O_2 concentrations were 0.580 μM (IQR 0.244–1.722) in children who used Ecoscreen and 0.180 μM (IQR 0.117–0.412) in the 12 children who used Turbo-Deccs (p = 0.212). At visit 2, EBC H_2O_2 concentrations in asthmatics obtained with Turbo-Deccs or Ecoscreen were significantly higher than in healthy control children (p = 0.006 and 0.01, respectively).

Table 3 shows the comparison of exhaled H_2O_2 concentrations between children receiving inhaled corticosteroids as long-term treatment and those who did not. At visit 2, exhaled H_2O_2 concentrations were significantly higher in children with auscultatory wheezing (0.18 μM, IQR 0.11–0.31) and in those free of auscultatory wheezing (0.71 μM, IQR 0.13–0.88) compared with controls (p = 0.034 and $p < 0.001$, respectively). However, there was no difference in H_2O_2 levels between asthmatics with auscultatory wheezing and those without (p = 0.061). Furthermore, in asthmatics with or without auscultatory wheezing, H_2O_2 levels were not statistically different from those detected at the first visit. At the second visit, there was no significant difference in H_2O_2 concentrations between asthmatics who received systemic steroids for exacerbation and those who did not (p = 0.224). There was no significant correlation between EBC H_2O_2 concentrations and O_2 saturation (at baseline, r = 0.344, p = 0.117; at visit 2, r = –0.96, p = 0.671) or symptom-drug score (at visit 2, r = –0.126, p = 0.577).

Pulmonary Function

At visit 1, in asthmatic children, pulmonary function values were significantly lower than those of healthy control children (table 4). At visit 2, asthmatic children had FEV_1 % predicted values similar to those of controls (table 4). No significant differences were found in lung function values between baseline and visit 2 (table 4). Both at visit 1 and visit 2, there was no correlation between FEV_1 % predicted values, $FEF_{25–75}$ % predicted values, FVC % predicted values and H_2O_2 levels, O_2 saturation or symptom-drug score. At the second visit, both in children with auscultatory wheezing and in those free of symptoms, no correlation was found between H_2O_2 levels and FEV_1 % predicted values, FVC % predicted values, $FEF_{25–75}$ % predicted values.

Table 4. Lung function in controls and asthmatic children

	Control subjects (n = 12)	Asthmatic children (n = 22)	p value
FEV_1, % predicted			
Before treatment	112.5 ± 15.75	94.9 ± 11.94[a]	0.004
After treatment		101.4 ± 13.92[a]	0.135
$FEF_{25–75}$, % predicted			
Before treatment	106.17 ± 19.75	76.5 ± 18.25[b]	<0.001
After treatment		81.91 ± 19.33[b]	0.001
FVC, % predicted			
Before treatment	104.66 ± 14.33	84.36 ± 14.42[c]	0.001
After treatment		89.62 ± 15.77[c]	0.017

Data are presented as the mean ± SD.
[a] p = 0.105; [b] p = 0.481; [c] p = 0.33.

Discussion

The results of the present study show that mean levels of H_2O_2 in EBC are significantly higher in children with asthma exacerbation than in normal control subjects and remained elevated after a 7-day treatment [17].

In agreement with our results, Dohlman et al. [15] found that 3 out of 4 children with acute exacerbation had higher EBC H_2O_2 concentrations than controls. Our results extend previous findings by examining a greater number of patients. Furthermore, to our knowledge, this is the first report providing data on exhaled H_2O_2 concentration in the short-term follow-up of exacerbation. Dohlman et al. [15] reported no differences in exhaled H_2O_2 levels between asthmatic children with acute asthma and those with upper respiratory infections, not accompanied by asthmatic symptoms. Our study design does not permit to exclude that a coexistent upper respiratory infection may have enhanced H_2O_2 levels. Future research may clarify this issue. Our findings cannot be directly compared with earlier investigations of EBC H_2O_2 concentrations focused on asthmatics who were free of asthmatic symptoms. Consistent with previous observations, data showed significantly increased exhaled H_2O_2 levels in asthmatic children with [15] and without upper respiratory tract infections [14] as well as in adults [10, 16, 17] with asthma, even if there was an overlapping with the levels of controls [10, 14–16, 26]. At variance, Robroeks et al. [27] found no difference in EBC H_2O_2 levels between childhood asthmatics and controls. Different methods used to detect EBC H_2O_2 concentrations may explain these different findings.

EBC collection is not yet fully standardized and several issues need to be considered in interpreting concentrations of EBC constituents [2, 28]. For example, we followed the suggestion of collecting EBC 10 min or more after forced expiratory manoeuvers. However, there is no consensus in this recommendation [2]. At present, there is no evidence showing that changes in airway caliber cause any difference in mediator release or dilution of EBC [29, 30], but this question has not been studied systematically. Another issue is the flow dependence of exhaled H_2O_2 levels. We are aware that, in one study only, exhalation flow influences the level of exhaled H_2O_2; at higher flows, exhaled H_2O_2 concentration is lower, but with the low flows during tidal breathing, the effect is minor [31]. However, symptomatic children have higher breathing rates and may have higher minute ventilation, which could theoretically influence the results. We did not take into consideration the control of minute ventilation as it is not requested by the recommendation [2]. We believe that if a bias could have been present because of that, it could probably have randomly affected the sample. We think that this factor deserves attention, and therefore, we would like to standardize exhalation flow ventilation in the next studies, as shown by Franklin et al. [32].

Another concern is the method employed to measure EBC H_2O_2. We used the fluorometric method [33, 34], even if the lack of established reference values has not permitted to ascertain sensitivity and specificity and has hampered the validation of measurements [35]. However, data reproducibility has been shown in a previous study by Goldoni et al. [20].

Only speculative hypothesis may be offered to explain the reason for which treatment of asthma exacerbation does not affect exhaled H_2O_2 levels. In asthmatic adults, EBC H_2O_2 is related to inflammatory cells, especially the number of eosinophils in the sputum [16]. Furthermore, it is associated with neutrophils in mild but not in moderate asthma [10]. A meta-analysis of EBC H_2O_2 levels in stable asthmatic patients [13] free of symptoms included 8 heterogeneous cross-sectional trials. It provided indicative evidence that patients treated with inhaled corticosteroids had exhaled H_2O_2 concentrations which were significantly lower than those in steroid-untreated patients. Furthermore, both steroid-treated and -untreated asthmatics had higher levels of EBC H_2O_2 than healthy subjects [13]. Along this line, Antczak et al. [36] showed that in asthmatic children, inhaled beclomethasone significantly reduced expired H_2O_2 levels in comparison with placebo. These findings may be explained by the an-

ti-inflammatory properties of corticosteroids which inhibit an oxidative burst of leucocytes and eosinophil recruitment to the airways. However, Horvath et al. [16] showed that asthmatic adults whose symptoms were not controlled by inhaled steroids had increased levels of EBC H_2O_2 that were not significantly different from those in stable steroid-naive asthmatics. Accordingly, we found that there was no relationship between systemic corticosteroid administration and EBC H_2O_2 levels during acute asthma exacerbations. These findings support the suggestion that corticosteroid succeeds in suppressing only a part of the airway inflammation [37]. Therefore, we may hypothesize that in acute exacerbation oxidative stress may continue after health restoration possibly because of persistent inflammation in the airways despite steroid treatment. However, the small number of children treated with systemic corticosteroids and the design of the study do not consent to reach firm conclusions. On the other hand, it is possible that lack of variation in EBC H_2O_2 levels may depend on the short time between EBC collections.

The issue arises whether there is a correlation between FEV_1 values and exhaled H_2O_2 levels in asthmatic children. We have found that bronchodilator treatment was effective in producing relief of airway obstruction but not in changing exhaled H_2O_2 levels. Therefore, we believe that H_2O_2 levels do not reflect changes in airway caliber despite the fact that H_2O_2 induces contraction of respiratory smooth muscles [38]. Our findings are in agreement with prior reports showing no correlation between FEV_1 and H_2O_2 both in healthy [39] and asthmatic children [15] as well as in adults [16]. On the contrary, Loukides et al. [10] found a significant inverse correlation between FEV_1 values and H_2O_2 levels in steroid-naive adults with moderate asthma but not in those treated with inhaled steroids. On the other hand, Antczak et al. [36] found an inverse correlation between FEV_1 and H_2O_2 in children treated with inhaled beclomethasone. Variations in study design may explain our different findings. Also, the severity of asthma may play a role. In our study, we included children with acute exacerbation who did not require admission. In these children, FEV_1 values are poorly correlated with the degree of wheezing, clinical score and oxygen saturation [40]. Our findings of quite high lung function values may be explained by the fact that patients were probably visited at the onset of exacerbation and symptoms usually precede the lung function decline [41].

A possible limitation of this study was that EBC was collected with two different devices. Two different condensers were available: Turbodeccs and Ecoscreen were used in children enrolled in Parma and in Milan, respectively. Previous studies have shown that data of Ecoscreen [42] and Turbo-Deccs [20] are both reproducible, but direct comparison between the two condensers by measuring H_2O_2 in EBC collected by the two systems in the same subject has not been done in the present study. However, the risk of bias was considered acceptable for many reasons. First, there is no clear evidence that H_2O_2 levels may be influenced by condensing devices, probably due to its partial volatility. In fact, in a previous study, Rosias et al. [42] have found that median H_2O_2 concentrations and reproducibility did not significantly differ between four different condensers. Regarding other substances, comparison of pH values of fluid collected from two devices (Ecoscreen and RTube) has yielded conflicting results, and therefore, neither device is preferred over the other [43]. Second, it has been shown that the cooling temperature influences levels of EBC constituents. It has been found that there was a statistically significant difference in H_2O_2 levels comparing the cooling temperature +5 and –5 °C but not comparing –5 and –10°C. [20]. In the Turbo-Deccs, the cooling temperature is dysplayed and it was set at –5°C. The cooling temperature of Ecoscreen is not dysplayed, but it has been reported to be from –10 to –20°C. Therefore, we believe that a bias was not introduced by cooling temperature. Along this line, in our study, no statistically significant difference was observed comparing exhaled H_2O_2 levels from the two condensers used. However, we cannot exclude that a significant difference may be reached with a larger group of patients. Third, in consideration of the lack of recommendations on the use of different condensers, we think that real-life situations are necessary to confirm or dismiss these unsolved points. Finally, the behavior of H_2O_2 in EBC cannot be explained on the basis of its chemical and physical properties, and the most probable explanation may be that some was produced by a radical reaction in the gas phase or during the condensation process in water, irrespectively of the devices used [44].

Another limitation of our study is the lack of randomization and of a placebo group. However, it was neither ethical nor feasible to have a placebo group in this study. This weakness is partly balanced by the fact that physicians who visited children or performed spirometry were unaware of H_2O_2 results. An additional limitation is the heterogeneity of our population, especially with regard to the fact that both children who received inhalant steroid as maintenance treatment and steroid-naive patients were included in the study. Nonetheless, our data showed that H_2O_2 concentrations in EBC were not influenced by

pre-exacerbation treatment. Therefore, we think that a bias was not introduced. However, in this study, differences among asthmatic subpopulations cannot be properly assessed because subgroup comparisons not only suffer from the limitation of any observational study but also from those due to small sample size and lack of randomization. Therefore, our results on subpopulations should be considered suggestive and may only provide directions for future research.

A potential weak point is H_2O_2 volatility. It is clear that H_2O_2 has a relatively high Henry's constant in comparison with the other substances, and so its volatility should be low (although not negligible) at temperatures of 25–37°C. In a previous study, the volatility of H_2O_2 ex vivo was less than that of water on the basis of the differences in EBC H_2O_2 content observed with variations in condensation temperature [20]. Moreover, in vitro experiments demonstrate that H_2O_2 is only slightly volatile in a standard solution at 37°C under a constant flow of air saturated with water vapor (only 1.3% evaporated after 3 h) [44]. Therefore, with the systems used, we cannot guarantee a complete recovery of all H_2O_2 which is exhaled and which is produced during the exhalation. However, they represent a good compromise between efficiency in collecting EBC and the stability of the biomarkers within it, because of the more efficient condensation of water obtained by using very low temperatures.

In conclusion, our study has shown that H_2O_2 levels in EBC are significantly elevated in children with acute asthma exacerbation. In the short-term follow-up, exhaled H_2O_2 levels were not correlated with lung function and remained elevated despite the improvement in symptoms.

Therefore, H_2O_2 seems not to be a useful marker to investigate acute asthma exacerbation.

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