

Original article

3-Nitrotyrosine, a marker of nitrosative stress, is increased in breath condensate of allergic asthmatic children

Background: Asthmatic patients have high exhaled nitric oxide (NO) levels. NO-mediated inflammatory actions are mainly due to NO conversion into reactive nitrogen species, which can lead to nitrotyrosine formation. The aim of this study was to assess 3-nitrotyrosine (3-NT) levels in exhaled breath condensate (EBC) of asthmatic and healthy children and to investigate whether there is any relationship with exhaled NO (FE_{NO}) and lung function.

Methods: The study included 20 asthmatic children (10 steroid-naïve with intermittent asthma, 10 steroid-treated with unstable persistent asthma) and 18 healthy controls. They underwent FE_{NO} measurement, EBC collection and spirometry. 3-NT was measured by a new liquid chromatography–tandem mass spectrometry (LC-MS/MS) method in isotopic dilution.

Results: The median EBC concentration of 3-NT (expressed as nitrotyrosine/tyrosine ratio × 100) in asthmatic children was fivefold higher than in healthy subjects [0.23% (0.12–0.32) vs 0.04% (0.02–0.06), *P* < 0.001] with no difference between steroid-naïve and unstable steroid-treated asthmatic patients. FE_{NO} levels were higher in asthmatic [44.6 ppb (36.0–66.0)] than in healthy children [7.5 ppb (6.0–8.8), *P* < 0.001]. No correlation was found among 3-NT, FE_{NO} and lung function parameters.

Conclusion: Nitrotyrosine is high in EBC of asthmatic children and could be considered as a noninvasive marker of nitrosative events in the airways.

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Key words: asthma; exhaled breath condensate;
exhaled nitric oxide; liquid chromatography–mass
spectrometry; nitrotyrosine.

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Accepted for publication 27 August 2005

Oxidative and nitrosative events have a crucial role in asthmatic lung disease (1). Nitric oxide (NO) levels are high in the exhaled air of allergic asthmatic patients. An increased production of NO in inflammatory-immune processes of the respiratory tract is assumed to be a host defense mechanism. High levels of NO can also cause respiratory tract injury, however, and thus contribute to the pathobiology of respiratory tract disease. This dual property of NO can be explained by the observation that NO is involved in different biochemical pathways, depending on its concentration and on local redox status (2).

Oxidative stress increases in asthma (1). Eosinophils and the other inflammatory cells recruited in the asthmatic airways produce large amounts of reactive oxygen species (ROS) (1). In asthmatic subjects, for instance, bronchoalveolar lavage superoxide (O₂^{•−}) concentrations

rise and correlate negatively with FEV₁ (3). Moreover, O₂^{•−} and other ROS can react with various molecules, forming more potent cytotoxic radicals (4). The reaction of O₂^{•−} with NO yields the powerful oxidant peroxynitrite (ONOO[−]). This radical has been shown to induce hyper-responsiveness in guinea pig airways (5), to inhibit pulmonary surfactant (6), and to damage pulmonary epithelial cells (6). Peroxynitrite can cause lipid peroxidation, protein nitration and sulfhydryl oxidation, and it is considered largely responsible for most of the adverse effects of excessive NO generation.

The amino acid tyrosine appears to be particularly susceptible to nitration and the formation of 3-nitrotyrosine (3-NT) may represent a biomarker for the generation of reactive nitrogen intermediates *in vivo* (7). Nitration of tyrosine residues seems to inactivate numerous enzymes and prevent kinase substrate phosphorylation, suggesting that tyrosine nitration does not only give rise to inactive ‘footprints’ of nitrosative stress, but may also have a functional relationship with the pathobiology of inflammatory disease (2).

In bronchial biopsies from asthmatic patients, a strong immunoreactivity has been demonstrated for nitrotyrosine in the airway epithelium, lung parenchyma and

Abbreviations: 3-nitrotyrosine, 3-NT; Exhaled breath condensate, EBC; Fractional exhaled nitric oxide, FE_{NO}; Inhaled corticosteroids, ICS; Liquid chromatography–tandem mass spectrometry, LC-MS/MS; Peroxynitrite, ONOO[−]; Reactive nitrogen species, RNS; Reactive oxygen species, ROS; Solid phase extraction, SPE; Superoxide, O₂^{•−}.

inflammatory cells (8, 9). Nitrotyrosine has also proved to be detectable in the exhaled breath condensate (EBC) of both healthy subjects and asthmatic patients, and increased nitrotyrosine levels have been reported in the EBC of adult patients with mild asthma (10). EBC is a noninvasive method that provides access to volatile and nonvolatile compounds and its composition appears to reflect airway lining fluid (11).

The aim of this study was to evaluate the levels of 3-NT in EBC of asthmatic and healthy children using a new liquid chromatography–tandem mass spectrometry (LC-MS/MS) method that is considered a reference analytical technique for determination of organic compounds in aqueous matrix. Any relationship between 3-NT and exhaled NO and lung function was also investigated.

Methods

Patients

The study included 20 asthmatic children (10 steroid-naïve with intermittent asthma and 10 inhaled corticosteroid (ICS)-treated with unstable persistent asthma) (Table 1) and 18 healthy controls. None of the children was a smoker. Asthmatic children were recruited among patients attending the Pulmonology/Allergy outpatients clinic at the Pediatrics Department in Padova. The diagnosis of asthma was based on clinical history and examination, pulmonary function parameters and response to β_2 -agonist agents, according to international guidelines (12).

Asthmatic children with intermittent disease had received no ICS treatment for at least 1 month.

Children with unstable asthma had needed urgent medical attention for asthma at least once and more than three oral steroid bursts in the last year. They had been on medium-to-high doses of ICS and β_2 long-acting agonists for at least 3 months and they had been regularly followed up at our center for at least 2 years. They received no oral steroids for at least 1 month before the study.

All asthmatic children were atopic, sensitized to common allergens (*Dermatophagoides pteronissinus* and *D. farinae*, grass pollen, *Artemisia vulgaris*, *Alternaria*, *Parietaria*, dog and cat), as evaluated by skin prick test. None of the children with grass pollen allergy were studied during the grass pollen season. Asthmatics were refrained from taking β_2 -agonists for at least 24 h before the test. Children who had acute upper or lower airway infection in the last 3 weeks, or who had a history of poor compliance, were excluded from the study. After being examined by a physician, the children

underwent fractional exhaled nitric oxide (FE_{NO}) measurement, EBC collection and spirometry. FE_{NO} measurement was performed before EBC collection and spirometry, and all of them were performed at least 1 h after the last food intake.

As a control group we enrolled 18 healthy children. They were Caucasian children with no history of asthma or atopy and they had suffered from no respiratory infections in the previous 4 weeks. None of the control subjects were taking any medication. The Ethics Committee of our hospital reviewed and approved the protocol and all parents gave their informed consent.

Exhaled breath condensate collection

Exhaled breath condensate samples were collected in a condensing device composed of two glass chambers (Incofar Srl, Modena, Italy), as previously described (13). The inner glass chamber was cooled with ice and suspended in a larger, outer glass chamber. The children, with no nose clip, were instructed to breathe tidally through their mouths through a two-way nonrebreathing valve for 15 min. To minimize salivary contamination the two-way valve served as a saliva trap. In addition a 12 cm banded tube vertically set connected the mouthpiece to the condensing device, with the child's mouth remaining in a lower position with respect to the device's inlet. Children were also asked to swallow their saliva periodically. Inhalation and exhalation through the mouth were checked by the movement of the inspiratory and expiratory valves. The collected EBC samples were then stored immediately in disposable polypropylene sterile tubes (Eppendorf) at -70°C . The glass chambers were cleaned with distilled water.

Exhaled nitrotyrosine measurement

Nitrotyrosine was measured by LC-MS/MS in isotopic dilution. The D₄-tyrosine was purchased from Cambridge Isotope Laboratories and ¹³C₆-tyrosine from Isotec Inc. (Miamisburg, OH, USA). The ¹³C₆-L-nitrotyrosine was synthesized in our laboratory according to Jiang and Balazy (14). Nitrotyrosine and nitronium tetrafluoroborate (NOBF₄) was purchased from Sigma-Aldrich (St Louis, MO, USA). All reagents were of HPLC grade.

Sample preparation

We added 100 μl of internal standard (D₄-tyrosine 4 μM and ¹³C₆-nitrotyrosine 200 nM in water) to a volume of 500 μl EBC. Five hundred microliters of HCl 100 mM were added to the sample and then samples were loaded in the solid phase extraction (SPE) Cartridges previously primed. Washing was done with 1 ml of HCl

Table 1. Subjects' characteristics, EBC 3-nitrotyrosine values, exhaled NO and spirometric parameters

	Healthy controls	Intermittent asthma (steroid naïve)	Unstable asthma (ICS-treated)
Number (males)	18 (12)	10 (8)	10 (6)
Age (years)	10 (6–13)	11 (5–15)	11 (8–16)
EBC 3-nitrotyrosine/tyrosine (%), median (IQR)	0.04 (0.02–0.06)	0.23 (0.11–0.33)**	0.24 (0.13–0.32)*
Exhaled NO (ppb), median (IQR)	7.5 (6.0–8.8)	46.6 (28.6–59.3)**	42.4 (36.0–66.0)**
FEV ₁ (%pred), mean $\pm$ SEM	99 $\pm$ 4	93 $\pm$ 6	92 $\pm$ 3
FEF _{25–75} (%pred), mean $\pm$ SEM	98 $\pm$ 7***	94 $\pm$ 5	78 $\pm$ 4
FEV ₁ /FVC ratio (%), mean $\pm$ SEM	88 $\pm$ 2***	88 $\pm$ 1***	82 $\pm$ 2

* $P < 0.01$ vs healthy children.

** $P < 0.001$ vs healthy children.

*** $P < 0.05$ vs unstable asthma.

IQR, interquartile range.

100 mM and elution with 400 μ l of ACN 15%. The eluted solution was subsequently butylated by evaporating to dryness and adding 200 μ l of butanol and 10 μ l of acetyl chloride at 60°C for 20 min. At last samples were evaporated to dryness and reconstituted in 50 μ l of ACN 20%. Analysis was performed with a Micromass Quattro Ultima Mass Spectrometer (Waters, Milford, MA, USA), connected with a Phoenix 40 FISIONS Instruments (Thermo Finnigan, San Jose, CA, USA) and an Alliance HT Waters 2795 HPLC system (Waters). A Waters switching valve was used for the enrichment on a Symmetry ShieldTM RP18 3.5 μ m Guard Column. Separation was performed with an XTerraTM MS C₁₈ 3.5 μ m 1.0 $\times$ 150 mm Column XTerraTM MS, with a C₁₈ 3.5 μ m 2.1 $\times$ 10 mm Guard Column as the precolumn. Samples were injected (10 μ l) with Alliance HT and loaded in the enrichment column (Symmetry ShieldTM). Acquisition was done in SRM, following these transitions: 238 > 136 for tyrosine (cone 70 V, coll. 15 eV), 242 > 140 for D₄-tyrosine (cone 70 V, coll. 15 eV), 283 > 181 for nitrotyrosine (cone 90 V, coll. en. 20), 289 > 187 for ¹³C₆-nitrotyrosine (cone 90 V, coll. 20 eV) and 286 > 184 for artifactual nitrotyrosine (cone 90 V, coll. 20 eV). Calibration curves were linear within the range 0.025–10 μ M for tyrosine and 3–200 nM for nitrotyrosine. The detection limit for nitrotyrosine after SPE was 0.2 nM on condensate. As shown in Figs 1 and 2, we checked whether free tyrosine nitration occurred during sample preparation (if this happens, it causes an overestimation of nitrotyrosine content), thus ensuring that no nitrotyrosine was produced artifactually.

The reproducibility and accuracy of the procedure were expressed as a coefficient of variation (CV%) and were determined from 10 independent analyses. The CV for a nitrotyrosine 30 nM solution was 3.5% and the accuracy was 2.2%. The recovery of nitrotyrosine 30 nM, spiked on breath condensate, was $\geq$ 98.5% and the accuracy was 2.6%.

To assess the repeatability of the measures, EBC was collected twice (24 h apart) in nine subjects for repeated nitrotyrosine measurements.

Fractional exhaled nitric oxide (FE_{NO}) measurement

Fractional exhaled nitric oxide was measured with the NIOX system (Aerocrine, Stockholm, Sweden) using a single-breath on-line method according to ERS/ATS guidelines for FE_{NO} measurement in children (15). Children inhaled NO-free air and exhaled through a dynamic flow restrictor with a target flow of 50 ml/s for at least 6–7 s.

Pulmonary function test

Pulmonary function parameters (FVC, FEV₁, FEF_{25–75}) were measured with a 10-l bell spirometer (Biomedin, Padova, Italy) and the best of three maneuvers was expressed as a percentage (%) of

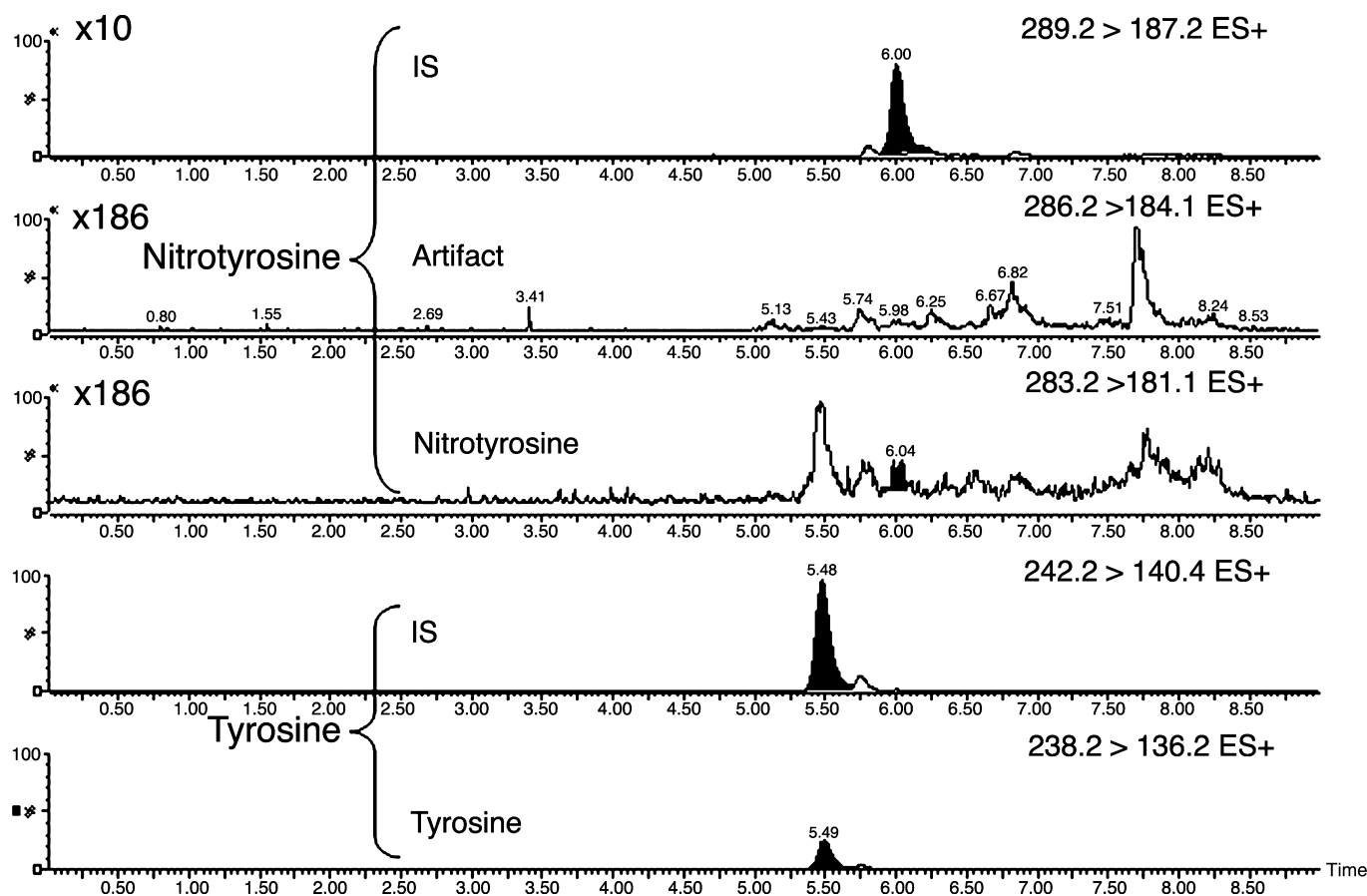


Figure 1. Chromatograms obtained from the exhaled breath condensate sample of a healthy subject. Peaks are normalized with respect to tyrosine internal standard. As shown, detected peaks are filled. The chromatograms show no presence of artifactual nitrotyrosine and very low amount of sample nitrotyrosine.

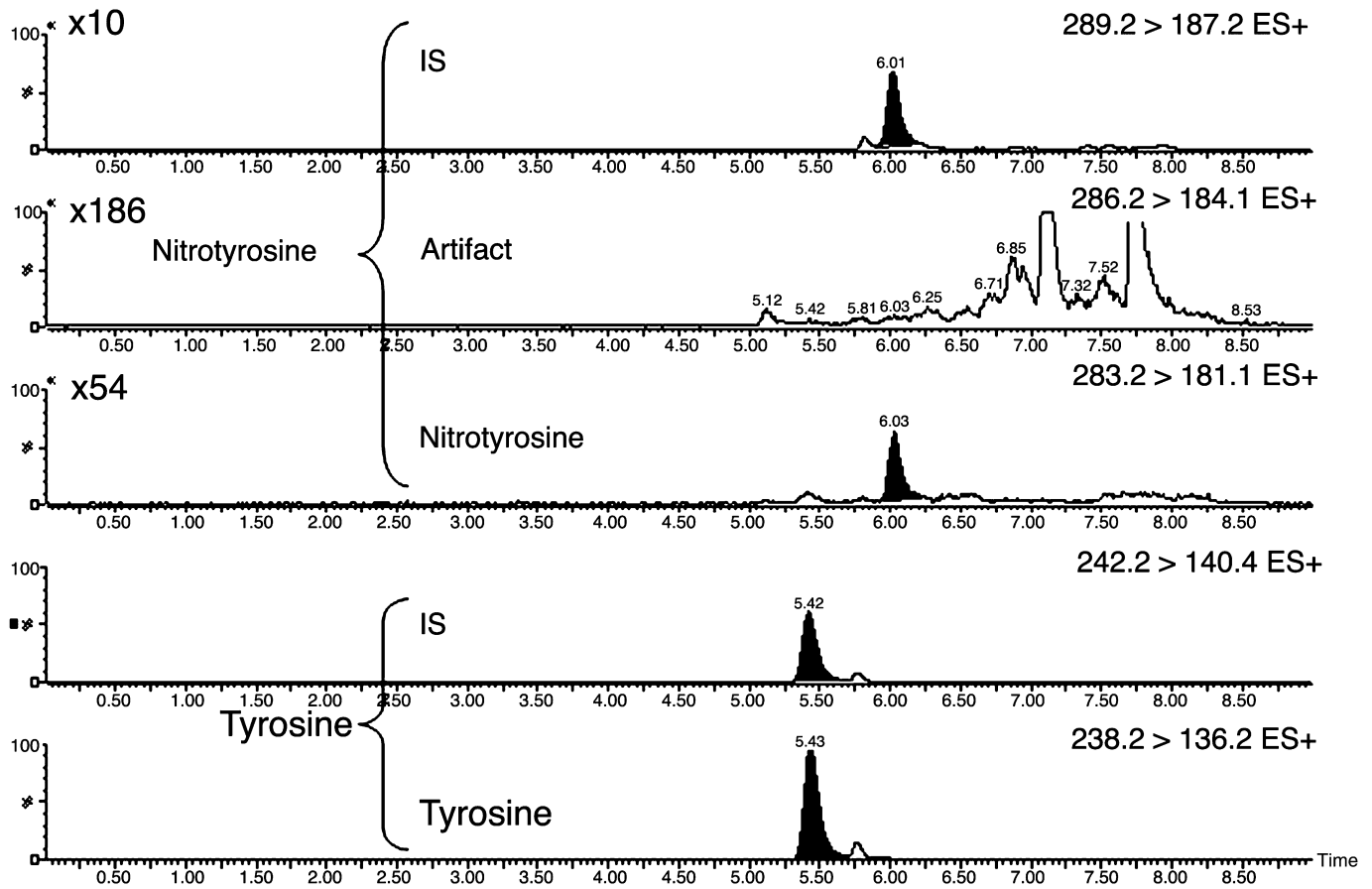


Figure 2. Chromatograms obtained from the exhaled breath condensate sample of an asthmatic patient. Peaks are normalized with respect to tyrosine. As shown, detected peaks are filled. The chromatograms show no presence of artifactual nitrotyrosine, while sample nitrotyrosine is clearly detectable.

predicted reference values. Accuracy of the spirometer was regularly verified with a calibrated syringe.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) for normally-distributed data and as median and interquartile range (IQR) for not normally-distributed data. For the latter (FE_{NO} and 3-NT), differences between groups were analyzed using the non-parametric Kruskal–Wallis test, followed (where significant) by the Mann–Whitney *U*-test for comparisons between groups. Correlations were evaluated by Spearman's rank test. Results were considered significant at a value of $P < 0.05$.

Results

3-Nitrotyrosine was detected in all EBC samples of asthmatic children, and in 17 of 18 EBC samples of healthy subjects.

Nitrotyrosine levels are expressed as the ratio of 3-NT nM/tyrosine nM $\times 100$. The EBC concentration of 3-NT was significantly higher in asthmatic children than in the healthy subjects [steroid-naïve asthmatics median 0.23%

(IQR 0.11–0.33), $P < 0.001$; ICS-treated unstable asthmatics 0.24% (0.13–0.32), $P < 0.01$; healthy controls median 0.04% (0.02–0.06)] (Fig. 3, Table 1). There was no significant difference in 3-NT concentration between the two groups of asthmatic children.

The coefficient of repeatability (i.e. two standard deviations of the overall average difference) was 0.042% for 3-NT measurements, with all the individual points on the Bland–Altman plot falling within this range.

The FE_{NO} levels were significantly higher in both steroid-naïve [median 46.6 ppb (IQR 28.6–59.3), $P < 0.001$] and ICS-treated asthmatic children [42.4 ppb (36.0–66.0), $P < 0.001$] than in healthy controls [7.5 ppb (6.0–8.8)] (Table 1). No significant differences in FE_{NO} levels were found between the two groups of asthmatic children ($P = 0.91$).

The FEV_1/FVC ratio was significantly lower ($P < 0.05$) in the ICS-treated unstable asthmatic children than in the steroid-naïve cases or healthy controls. FEF_{25-75} was also significantly lower ($P < 0.05$) in the ICS-treated unstable children than in the healthy controls, while there was no difference in FEV_1 between the three groups (Table 1).

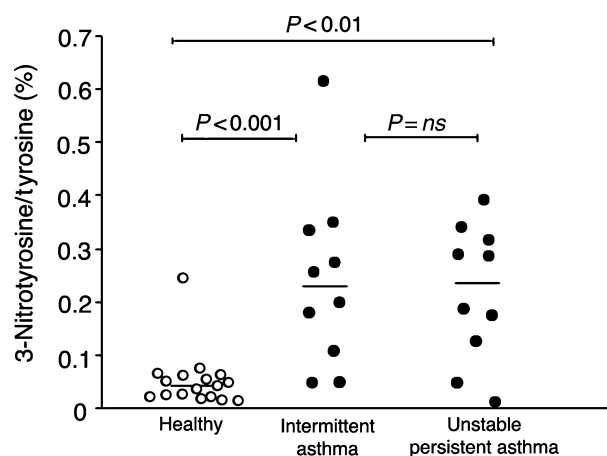


Figure 3. Nitrotyrosine levels in exhaled breath condensate of unstable asthmatic children treated with inhaled corticosteroid, steroid-naïve, stable asthmatic children and healthy controls. Horizontal bars represent median values.

No correlation was found between 3-NT EBC and FE_{NO} either in the whole group of asthmatic children ($P = 0.68$) or in the two subgroups of asthmatic children ($P = 0.75$ and $P = 0.29$ respectively). No significant correlation was found between spirometric parameters and 3-NT concentrations or FE_{NO} levels.

Discussion

Nitrotyrosine is thought to be a marker of reactive nitrogen species (RNS) formation in the airways. The results of our study show that its formation is greater in the EBC of asthmatic children, indicating active RNS production in their airways. To our knowledge, this is the first study to evaluate airway protein nitration by LC-MS/MS applied to EBC.

Previous studies have reported on the possible role of nitrotyrosine in the mechanisms of asthmatic airway inflammation. Using a semi-quantitative method, Saleh et al. (8) found a strong immunoreactivity for nitrotyrosine and inducible NO synthase in airway epithelium and inflammatory cells of bronchial biopsies from asthmatic patients. Hanazawa et al. (10) reported increased nitrotyrosine levels measured by enzyme-linked immunosorbent assay (ELISA) in the EBC of adult asthmatic patients. Recently, Mac Pherson et al. (16) demonstrated that eosinophils have an important role in generating NO-derived oxidants and they actively contribute to oxidative protein modification in asthmatic lung and airways.

Increased NO and O_2^- production have been implicated in the pathogenesis of asthma (17). Nitric oxide rapidly reacts with O_2^- in the airways, forming $ONOO^-$, which is responsible for most of the cytotoxic effects of NO (18, 19). In addition, $ONOO^-$ can directly cause airway hyper-responsiveness (5) and correlates with hyper-responsiveness in asthmatic patients (8).

3-Nitrotyrosine is a stable marker of protein nitration, a biological process resulting in the addition of a nitro group to tyrosine. Nitrotyrosine derives from the biochemical interaction of $ONOO^-$ with tyrosine (20), but also alternative pathways can lead to its formation (16, 21). Whether it is due to the effects of $ONOO^-$ or of other NO-derived oxidants, the *in vivo* expression of nitrotyrosine is a marker of oxidant injury.

Tyrosine nitration is not only a footprint of lung nitrosative stress, but could also have a deleterious impact on cell function and viability (21). Several studies have shown that tyrosine nitration may alter protein function. There is evidence that this process can inhibit protein phosphorylation by tyrosine kinases, thus interfering with the signal transduction mechanism (2). It has also been suggested that tyrosine nitration plays a part in regulating eosinophil chemotaxis by reducing RANTES and IL-5 binding to eosinophils (22). In addition, *in vitro* nitration of a specific tyrosine residue has been shown to inactivate manganese superoxide dismutase (23, 24); by causing a reduction in superoxide dismutase activity, this mechanism may lead to a greater concentration of ROS and RNS, with consequent tissue damage (21).

Our study found a value of 3-NT five times greater in asthmatic children than in healthy controls, but no differences between steroid-naïve and ICS-treated patients (Table 1, Fig. 3). In previous studies, nitrotyrosine immunoreactivity and EBC nitrotyrosine levels were lower in asthmatic patients treated with inhaled or oral corticosteroids (8, 10). On the contrary, Kaminsky et al. (9) demonstrated intense nitrotyrosine immunoreactivity in airway and lung parenchyma specimens from asthma patients who had died of status asthmaticus despite steroid treatment, suggesting that status asthmaticus is characterized by corticosteroids failing to control the formation of RNS.

We also measured FE_{NO} and found that ICS treated children with unstable asthma had median FE_{NO} levels similar to those of untreated patients (Table 1), as previously described in patients with difficult asthma (25, 26). The FE_{NO} is increased in patients with asthma and it is considered a marker of eosinophilic airway inflammation. As an indicator of responsiveness to anti-inflammatory treatment, FE_{NO} values have been shown to drop after steroid treatment, coinciding with better disease control. It has nonetheless been recognized that, for some patients with difficult asthma, corticosteroids may have little effect on symptoms, FE_{NO} and eosinophils, suggesting an heterogeneity in the sensitivity to ICS on the pathway of airway inflammation reflected by NO (26). Other inflammatory cells, e.g. neutrophils, may have a major role in unstable asthma, or there may be a noninflammatory basis for persistent symptoms (25). The wide range of FE_{NO} and 3-NT levels found in patients with unstable asthma despite ICS treatment suggests that these patients have distinct patterns of airway inflammation to account for their symptoms, supporting a hetero-

geneity of asthma inflammation and/or different individual responses to anti-inflammatory treatment.

In addition, we recognize that a limit of the present study is the lack of a group of children evaluated before and after a course of inhaled glucocorticosteroids. Although we found no significant differences in both 3-NT and FE_{NO} values between unstable ICS-treated and stable steroid-naïve subjects, we cannot exclude that these markers could have been higher in steroid-naïve children with unstable asthma.

We did not find a correlation between EBC nitrotyrosine and FE_{NO} in asthmatic children. It has been demonstrated that, beside the interaction of $ONOO^-$ with tyrosine, alternative pathways, involving leukocyte peroxidase, contribute to tyrosine nitration (16, 21). The contribution of different pathways to nitrotyrosine synthesis might explain the lack of a linear correlation between NO and nitrotyrosine.

Very few data are available on EBC nitrotyrosine measurement and are mainly based on ELISA. Using enzymatic immunoassay to measure EBC compounds may have drawbacks related to the fact that the concentrations of several mediators in EBC are low, too close to the detection limit of currently-available ELISA techniques. That is why more specific analytical methods should be considered. This study is the first to assess 3-NT quantitatively using a reference analytical method, LC-MS/MS, applied to EBC. The main advantage of this method is that we used a stable isotope dilution to quantify nitrotyrosine levels and potential artifacts. HPLC also enables a retention time

to be added as a further parameter for the identification. To improve the sensitivity, the nitrotyrosine was butylated before the analysis. A previously-described problem of the derivatization is that, being performed in acid conditions, the presence of nitrite and nitrate in the sample may lead to artifactual tyrosine nitration causing an overestimation of nitrotyrosine (27). To prevent this, we purified the sample by SPE to eliminate the salts (28), and added a specific analysis for monitoring artifactual nitrotyrosine. As $^{13}C_6$ -nitrotyrosine gives a different mass transition compared with d_3 -nitrotyrosine (deriving from nitration of the d_4 -tyrosine used as the internal standard for the tyrosine), there is no risk of overestimating the nitrotyrosine. A further advantage of LC-MS/MS is that, unlike enzymatic immunoassay, we can measure nitrotyrosine and tyrosine in the same run. Finally, normalizing nitrotyrosine to tyrosine may be useful as an internal reference for reducing the variability due to different collected volumes and dilutions of EBC but further studies are necessary to evaluate this methodological aspect.

In conclusion, we found that 3-NT formation is greater in the EBC of asthmatic children, indicating that nitrosative processes are more active in the lung of asthma patients. We suggest that applying LC-MS/MS to EBC can be a sensitive method for noninvasively measuring nitrosative events in the airways. Further studies are needed to establish whether augmenting lung antioxidant defenses could serve as an adjunctive strategy for treating asthmatic patients with evidence of greater oxidative stress.

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