

BREATH MARKERS OF OXIDATIVE STRESS AND AIRWAY INFLAMMATION IN SEASONAL ALLERGIC RHINITIS

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Oxidative stress (OS) is well documented in asthma, but so far little data has been reported in non-asthmatic patients with Seasonal Allergic Rhinitis (SAR). The aim of this study is to investigate the degree of OS and airway inflammation in patients with SAR, with and without concomitant Asthma (SAR +A), using breath markers in exhaled air and in Exhaled Breath Condensate (EBC). In addition, the effects of natural allergen exposure and intranasal steroid treatment on these markers were evaluated. Exhaled NO (eNO) and CO, combined with measurements of 8-Isoprostane (Iso-8), Leukotriene B₄ (LTB₄) and nitrate/nitrite in EBC, were performed in 23 patients, 11 with SAR and 12 with SAR+A, and 16 healthy subjects. Iso-8 and LTB₄ were significantly increased in both groups of patients (median values 43.6 pg/ml and 138.4 pg/ml in SAR group; 38.9 pg/ml, and 164.6 pg/ml in SAR+A group respectively; $p > 0.05$) compared to healthy subjects (18.6 pg/ml and 7.8 pg/ml; $p < 0.05$). Nitrate/nitrite and eNO levels were elevated in both groups compared to controls, but were significantly higher in the SAR+A compared to SAR group (nitrate/nitrite 9 μ M and 3.9 μ M; $p = 0.025$; and eNO 18.5 ppb and 12.5 ppb, respectively; $p > 0.05$). Nasal steroids caused significant reduction in LTB₄ and 8-isoprostane levels in both groups of patients ($p < 0.05$), while nitrate levels and eNO concentration were little affected by nasal treatment. OS markers were decreased at normal levels out of pollen season. Natural allergen exposure induces OS and airway inflammation, as assessed by measurements of markers in EBC and exhaled air, in patients with SAR who have no clinical signs of lower airway involvement. Besides, intranasal steroid treatment may have a regulatory role in the OS.

Epidemiological studies indicate that 60-85% of patients with asthma have concomitant allergic rhinitis, whereas up to 40% of patients with allergic rhinitis have clinically demonstrable asthma (1). Allergic rhinitis has been prospectively shown to be a major independent risk factor for asthma. Furthermore, several studies have shown that asthma and rhinitis share common inflammatory

processes although the exact pathophysiological interactions between upper and lower airways still remain unclear. Epithelial destruction, mast cell and eosinophil accumulation, T-lymphocyte activation and enhanced cytokine expression are observed both in the nasal mucosa and in bronchial biopsies in asthma (2). In addition, the detection and measurement of non-invasive inflammatory markers

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strongly support the existence of lower airway inflammation in allergic rhinitis both in and out of allergen season (3-5). These observations support the view that there is a close link between allergic rhinitis and asthma and these diseases may represent distinct manifestations of a unique inflammatory process in the airways that leads to the concept of "one airway-one disease".

Reactive oxidant generation is part of the normal metabolism of many types of cells and is critical for cell homeostasis. To protect itself against exposure to noxious oxidants, the lung has a well developed antioxidant system. There is ample evidence that respiratory disorders, such as asthma and chronic obstructive lung disease (COPD) are characterized by oxidative stress (OS); besides, it is thought to contribute to the pathogenic mechanism of chronic airway diseases, including asthma (6). OS may occur either as a response to environmental exposure (air pollution and cigarette smoke) or as a consequence of underlying inflammation (7). Several studies have shown that blood eosinophils and monocytes, as well as airway macrophages from asthmatic patients, produce more reactive oxygen species (ROS) compared to controls (8). More recently measurements of exhaled gases have shown increased ROS and some of their reaction products in patients with chronic airway diseases, such as asthma (9).

Exhaled breath condensate analysis has been introduced as a non-invasive sampling method to evaluate OS and inflammation in the lower airways (10). Hydrogen peroxide, F_2 -isoprostanes, carbon monoxide (CO), ethane, nitrate/nitrite, Leukotrienes E_4 and B_4 (LTB_4) have all been reported to be elevated in EBC samples of asthmatic patients compared to healthy individuals (11-12). Exhaled biomarkers such as cysteinyl-leukotrienes (cys-LTs) and 8-isoprostane have been correlated with disease severity (13). However, there are very few studies addressing OS in allergic rhinitis.

This study was undertaken in order to investigate breath markers of OS and airway inflammation in patients with seasonal allergic rhinitis with and without concomitant asthma. Breath analysis included combined measurement of exhaled NO, CO and isoprostane-8 (ISO-8), LTB_4 , nitrates in EBC. Assessment of the influence of natural allergen

exposure and the effect of nasal treatment on the above-mentioned breath markers are the aims of this study.

MATERIALS AND METHODS

Study population

Twenty-three patients with a history of SAR for the last two years took part in the study. Patients were recruited from the allergy clinic and took part voluntarily in the study if they fulfilled the inclusion criteria. The protocol described below was approved by the Ethics Committee of "Evgenidion" Hospital and was conducted according to the Helsinki declarations; informed consent was obtained from each participant. At study entry, all patients were symptomatic: 11 had a history of SAR symptoms, while 12 reported history of SAR and concomitant seasonal asthma (SAR+A). The diagnosis of asthma was based on history, spirometric values and/or bronchial hyperreactivity tests ($PD_{20} < 2$ mg/ml methacholine). All patients were atopic with at least one positive prick test to common seasonal inhalant allergens (*Grasses sp*, *Parietaria judaica* or *Olea europea*).

A control group of 16 healthy non-atopic volunteers (controls) was also included in the study. None of the participants in the study had been treated with nasal or inhaled medications before enrollment in the study; all subjects were non-smokers and none had had symptoms of upper or lower respiratory infection during the month preceding the study.

Study design

A detailed history was obtained in order to define the study groups at study entry. At the baseline visit, skin prick testing, spirometry and methacholine challenge test were performed for assessment of bronchial hyperreactivity.

The study subjects visited the department three times according to the protocol:

The 1st visit was held in allergen season when patients with seasonal allergic rhinitis were symptomatic either with nasal symptoms alone or with concomitant asthma symptoms (baseline visit).

The 2nd visit took place after one month of treatment with nasal steroids (50 µg/ fluticasone propionate, 2 puffs per nostril once daily) (post treatment visit). During that month, patients with asthma were allowed to use short acting β_2 -agonists, as relief medication if asthma symptoms occurred.

The 3rd visit was made in 4-7 months, during a period of the year that was out of the pollen season. All patients were asymptomatic at this visit (off season visit).

Clinical assessment and nasal symptoms (sneezing, runny nose, nasal obstruction) or asthma symptoms (chest

tightness, dry cough or wheezing) were recorded at each visit. Measurements of eNO and eCO, and collection of EBC were also performed at each visit of the study.

Skin prick testing

Atopy was assessed in all subjects (patients and healthy volunteers) by skin prick tests, using a battery of 10 common inhalant allergens: *Dermatophagoides Pteronyssinus*, *Dermatophagoides Farinae*, mixed grasses I and II, *Parietaria sp.*, *Olea Europea*, mixed weeds, cat dander, dog epithelium, mixed moulds specimen containing *Aspergillus*, *Alternaria* and *Candida*; positive (histamine hydrochloride 10 mg/dl) and negative control (normal saline) were also used (HAL, allergen Lab B. V., Haarlem, Netherlands). A wheal diameter >3 mm was considered as criterion of positive skin prick test.

Pulmonary functions and bronchial hyperreactivity tests

A water sealed spirometer (Biomedin, Milan, Italy) was used for measuring FEV₁ and FVC. The best of three maneuvers was expressed as a percentage of the predicted value. Bronchial hyperreactivity was assessed by a methacholine provocation test. The methacholine solution was nebulized with a hand-held nebulizer (Dosimeter MB3, MEFAR Bovezzo, Italy). The challenge test was continued up to the dose of methacholine that caused a 20% drop of the baseline FEV₁ value or up to the point where the maximum dose was inhaled. The 20% decrease in FEV₁ compared to baseline value, was considered as criterion of positive provocation. The PD₂₀ was calculated automatically by extrapolation of the logarithmic dose response curve as the cumulative dose causing a 20% fall in FEV₁.

Exhaled NO and CO measurement

The measurement of NO concentration in the expired air in this study was performed in accordance with the recommendations of the European Respiratory Society (ERS) Task Force Report on standardization of equipment specification and respiratory maneuvers (14). Exhaled NO was measured by a chemiluminescence analyser (model LR2000, Logan research, Rochester, UK) sensitive to NO from 1 to 5000 ppb. The analyser was calibrated daily with NO/N₂ calibration gas containing 116 ppb. In addition to NO, this analyser measures eCO concentration, pressure and volume in real time.

Collection of exhaled breath condensate (EBC)

EBC was collected as described previously (9-13). Samples were collected using a commercially available condenser that allowed non-invasive collection of the non-gaseous components in the expired air (EcoScreen, Jaeger, Würzburg, Germany). Subjects breathed through a

mouthpiece with a two-way non-rebreathing valve, which also served as a saliva trap. They were asked to breathe at normal frequency and tidal volume, wearing a nose clip, for a period of 15 min. Nose clip use was mandatory in order to prevent any inhalation-exhalation through the nose during sample collection. The exhaled breath condensate, typically around 3 ml, was collected and aliquots were stored immediately at -80° C. All condensate samples were tested for salivary contamination by determining amylase activity spectrophotometrically (kinetic method) using the Enzymeline α -Amylase RTU (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. All samples tested for saliva were at the limit of detection, suggesting no contamination of breath condensate.

Measurement of 8-isoprostane (iso-8), Leukotriene B₄ (LTB₄) and nitrate/nitrite

The concentrations of 8-isoprostane (iso-8) and LTB₄ in EBC were measured by enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Both iso-8 and LTB₄ were measured within two weeks of EBC collection. All study samples were assessed and compared in the same experiment. The lower limits of detection for these assays were 3.9 pg/ml and 7.8 pg/ml, respectively. If iso-8 and LTB₄ were not detected in breath condensate samples, a value of 3.9 and 7.8 pg/ml was arbitrarily assigned. Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase, using the nitrate/nitrite Colorimetric Assay kit (LDH Method; Cayman Chemical, Ann Arbor, MI).

Statistical analysis

Due to the small sample size, non-parametric tests were used for the analysis of the data. Data were expressed as median values and interquartile range (25-75%). Differences between the subjects' groups were initially assessed by Kruskal-Wallis test and when a significant result was found, Mann-Whitney rank test was then performed. Comparisons between measurements at different time points within patient groups were assessed with the Wilcoxon signed rank test. Possible correlations of values of measured parameters at the same time points were assessed by Spearman's rank order test. Statistical analysis was not influenced by the imputation of values to the lower limits of detection since non-parametric tests used were based on ranks of values.

RESULTS

The subjects' characteristics are presented in Table I. There were no differences of baseline values

Table I. Subject characteristics.

	Healthy subjects	SAR	SAR+A
No of subjects	16	11	12
Age (yrs)	36 $\pm$ 8	35 $\pm$ 10	40 $\pm$ 11
Sex, (M/F)	3/13	7/4	4/8
FEV₁ (% pred)	99 $\pm$ 14	98 $\pm$ 15	97 $\pm$ 8
FVC (% pred)	101 $\pm$ 14	100 $\pm$ 14	98.5 $\pm$ 11
FEV₁/FVC	83 $\pm$ 8	81.4 $\pm$ 8	81 $\pm$ 4
BHR (methacholine)			
Positive/negative (+/-)	-	1/10	9/3

Data are expressed as mean values $\pm$ SD, unless stated otherwise.

SAR: seasonal allergic rhinitis

SAR+A: concomitant seasonal allergic rhinitis and asthma

FEV₁: forced expiratory volume in one second

FVC: forced vital capacity

BHR: bronchial hyperreactivity to methacholine

Table II. Exhaled breath markers in the study population during pollen season.

Exhaled breath marker	SAR n=11 median (range)	SAR+A n=12 median (range)	Healthy subjects n=16 median (range)
8-isoprostane (pg/ml)	44.0 (12.0-104.0)*	39.0 (4.0-125.0)*	19.0 (4.0-47.0)
LTB₄ (pg/ml)	138.0 (89.0-244.0)*	165.0 (37.0-248.0)*	7.8 (7.8-35.0)
Nitrate (μM)	3.9 (0-19.0)*	9.0 (0-22.0)*	2.0 (0-22.0)
NO (ppb)	12.5 (4.0-50.0)*	18.5 (9.5-51.0)*	3.8 (1.0-7.3)
CO (ppm)	5.0 (3.0-10.0)	5.4 (4.0-11.0)	4.5 (3.5-7.0)

Data are expressed as median values (interquartile ranges, 25th-75th percentiles).

SAR: seasonal allergic rhinitis

SAR + A: concomitant seasonal allergic rhinitis and asthma

NO: nitric oxide

CO: carbon monoxide

ppb: parts per billion, ppm: parts per million

$p < 0.05$, patients with SAR or patients with SAR+A vs healthy subjects

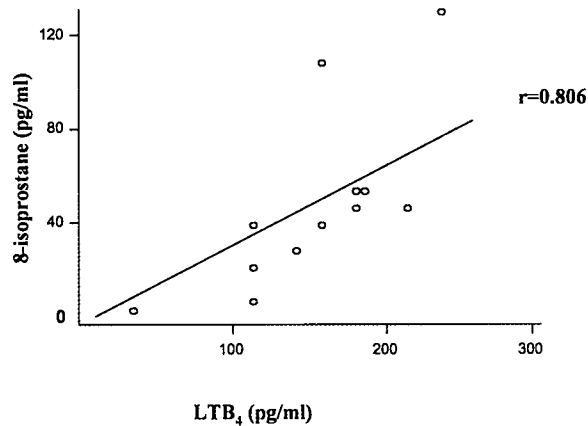


Fig. 1. Correlation between baseline values of iso-8 and LTB_4 concentrations in EBC in SAR + A patients ($r=0.806$, $p=0.002$, Spearman's rank order test). Individual data points ($^{\circ}$)

of FEV_1 and FVC between the groups. A positive response to methacholine challenge ($PD_{20} \leq 2$ mg/ml) was observed in 1 out of 11 patients with SAR and in 9 out of 12 patients with SAR+A.

All patients complied with nasal treatment and attended the scheduled second visit, while three of them were not included in the 3rd phase of the study, missing the 3rd visit out of season due to upper or lower respiratory infection that had occurred within the month preceding the scheduled visit. Measurement of exhaled markers (NO, CO) and breath collection was easily performed in all participants.

Measurements during pollen season (baseline visit)

Values of measured markers in Exhaled air and in EBC are shown in Table II. Iso-8 and LTB_4 levels were significantly increased in both groups ($p<0.05$) of patients (SAR and SAR+A), compared to healthy subjects. There were no significant differences between the two patient subgroups. In addition, nitrate/nitrite levels were elevated in both groups compared to controls, but nitrates levels were significantly higher ($p=0.025$) in EBC of patients with concomitant lower airway disease (SAR+A) than in patients with SAR. Similarly, exhaled NO was increased in both groups of patients in comparison with controls ($p<0.01$) with higher increase in patients with concomitant asthma

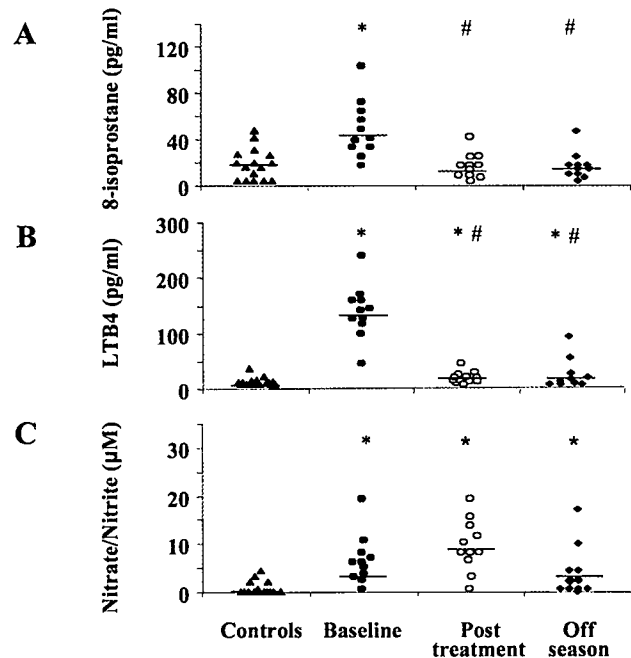


Fig. 2. Baseline individual and median values of (A) iso-8, (B) LTB_4 and (C) nitrite/nitrates concentrations in exhaled breath condensate, in healthy subjects (controls) and in SAR patients in pollen season (baseline), after intranasal steroid treatment, and off pollen season. Horizontal bar represents the median value.

* $p = 0.01$ compared to control subjects; Mann Whitney rank test.

$p < 0.05$ compared to baseline measurements; Wilcoxon signed rank test

(SAR+A). No differences were found in exhaled CO concentrations between the study groups. Baseline levels of iso-8 were positively correlated with LTB_4 levels in SAR+A patients' group (Fig. 1) measured in EBC ($r = 0.806$, $p=0.002$).

Measurements following one month nasal treatment with steroids (post treatment visit)

Breath analysis data following one month treatment with nasal steroids are presented in Fig. 2 for SAR and Fig. 3 for SAR+A group, respectively. One month treatment with nasal steroids, caused a significant ($p<0.05$) reduction of iso-8 in both groups of patients; 8.2 pg/ml (range 4-42 pg/ml) in patients with SAR and 18.5 pg/ml (range 4-37 pg/ml), in

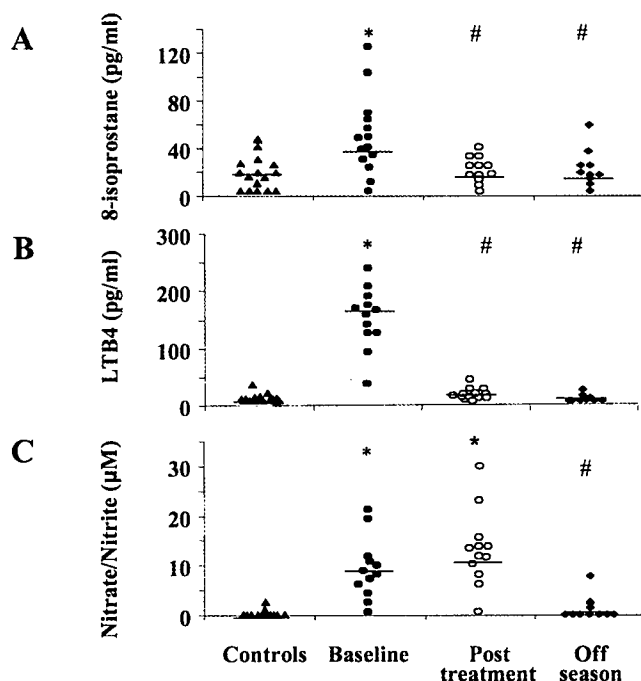


Fig. 3. Baseline individual and median values of (A) iso-8, (B) LTB_4 and (C) nitrate/nitrite concentrations in exhaled breath condensate, in healthy subjects (controls) and in SAR+A patients in pollen season (baseline), after intranasal steroid treatment, and off pollen season.

* $p = 0.02$ (8-iso, LTB_4), $p = 0.003$ (nitrate/nitrite) compared to control subjects; Mann Whitney rank test). Horizontal bar represents the median value.

$p < 0.05$ compared to baseline measurements; Wilcoxon signed rank test.

patients with SAR+A. In a similar way, significantly reduced values ($p < 0.001$) were also observed in LTB_4 both in SAR (16.5 pg/ml; range 13-39 pg/ml) and in SAR+A patients (21 pg/ml; range 8-44 pg/ml), after nasal treatment. On the contrary, no significant changes were observed in nitrate/nitrite levels in EBC in either group (10.3 pg/ml; range 0.6-20 pg/ml in SAR and 12 pg/ml; range 0-30 pg/ml in SAR+A, respectively). Similarly, there was no significant reduction caused by nasal treatment in exhaled NO levels in either group (15 pg/ml and 11 pg/ml, respectively) (Fig. 4A). Neither were any changes detected in exhaled CO (Fig. 4B).

Measurements during out of pollen season (off season visit)

Re-measurement of iso-8 during the 3rd visit (out of pollen season) revealed significant lower levels ($p < 0.05$) compared with baseline values in both SAR (13.2 pg/ml; range 3.9-46.6 pg/ml) and in SAR+A patients (14.2 pg/ml; range 7.8-59 pg/ml). Similarly, statistically significant lower LTB_4 levels ($p < 0.001$) were found in both groups (14.1 pg/ml, range 7.80-106.37 pg/ml in SAR and 7.8 pg/ml, range 7.80-20.85 pg/ml in SAR+A). No significant differences were found between the patient groups. On the contrary, no real changes were detected regarding nitrate/nitrite levels measured at this time point compared with baseline values measured at the 1st visit (in season).

Re-measurement of exhaled NO at this time point showed lower levels compared to baseline values in both groups (Fig. 4A). However, these eNO levels were significantly higher compared to baseline values of healthy controls ($p = 0.001$). No changes were detected in levels of exhaled CO (Fig. 4B).

DISCUSSION

In the present study, we have shown for the first time to our knowledge that breath markers of OS and lower airway inflammation are simultaneously detectable in EBC of patients with seasonal allergic rhinitis. These markers, ISO-8, LTB_4 and nitrates, were increased in symptomatic patients with SAR, irrespective of the existence of concomitant asthma symptoms during natural allergen exposure. These findings indicate that there is a strong link between upper and lower airways in patients with SAR and that underlying lower airway inflammation and OS may already exist even in the absence of clinical asthma. Furthermore, according to our results, intranasal corticosteroid treatment reduced OS markers, but did not influence nitrate/nitrite levels and exhaled NO concentrations.

There have been a growing number of studies indicating that specific quantification of isoprostanes in biological samples is a sensitive and a reliable non-invasive method allowing assessment of ROS-caused lipid peroxidation and OS *in vivo* (15). Isoprostanes are structurally stable isomers of prostaglandins and their *in vivo* production is primarily the result of free

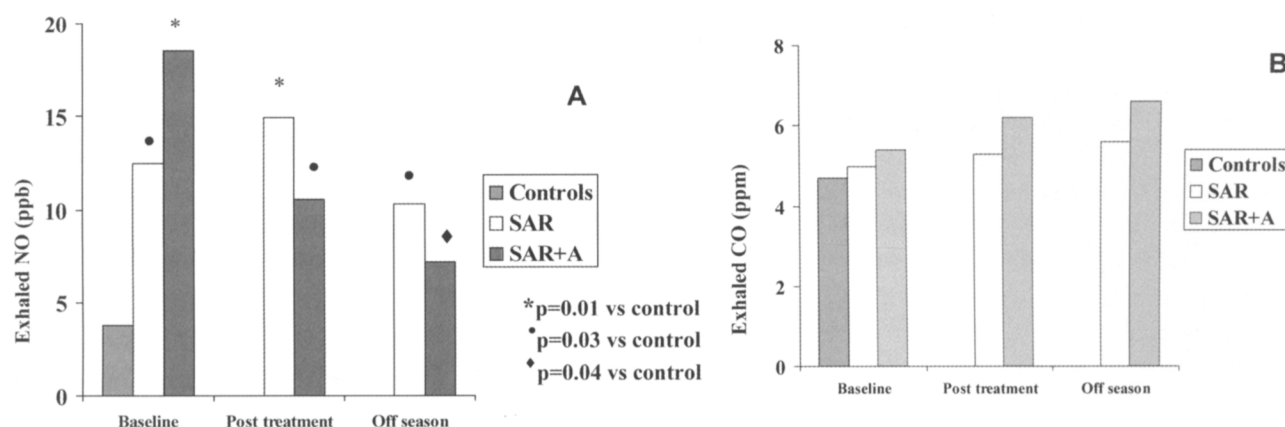


Fig. 4. Mean values of exhaled (A) NO and (B) CO concentrations in healthy subjects (controls); in SAR and SAR+A patients, in pollen season (baseline), after intranasal steroid treatment, and off pollen season.

* $p=0.01$ vs control, • $p=0.03$ vs control, ♦ $p=0.04$ vs control; Mann Whitney rank test

radical catalyzed peroxidation of polyunsaturated fatty acids. This process is a central feature of oxidative stress and contributes to pathophysiologic mechanisms of asthma (16). Besides, several *in vitro* studies have suggested an interaction between allergens and OS in the context of inflammatory lung disease (17). Our study supports this important role; natural allergic exposure in patients with SAR can cause OS and lower airway inflammation at comparable levels as in patients with concomitant asthma. An additional observation of our study is the significant correlation between iso-8 and LTB_4 levels in EBC samples. Also, in a previous study by Zanconato et al. higher levels were reported of cys-LTs and iso-8 in asthmatic children compared to healthy children (13).

LTE_4 and LTB_4 -like immunoreactivity in EBC were increased about 3-fold and 2-fold, respectively, in steroid naïve mild asthmatics compared with healthy controls (11). Furthermore, Csoma et al. reported increased exhaled LTB_4 levels in patients aged 7-14 yrs with mild and moderate to severe asthma compared with patients with mild intermittent asthma and normal subjects (18).

Increased levels of LTE_4 and LTB_4 were detected in EBC in non-asthmatic adult patients with persistent SAR during and after pollen season, compared to healthy subjects (3). Levels of both

breath-measured leukotrienes were decreased out of pollen season, but still remained higher compared to measurements in control subjects. In our study, LTB_4 levels were elevated in SAR patients, at comparable levels as in patients with seasonal allergic rhinitis and concomitant asthma, when measured during allergen season, but not out of season. A possible explanation for this discrepancy could be the fact that all participants in the study of Cap et al. had persistent moderate to severe rhinitis, while ours had seasonal rhinitis and were asymptomatic when measured out of pollen season.

eNO is considered a very useful non-invasive marker of lower airway inflammation, being increased in patients with asthma and decreased with anti-inflammatory treatment. eNO is also increased in atopic patients, even in the absence of asthma, while patients with allergic rhinitis have higher levels of eNO when compared with non-atopic subjects (19). In the present study, we re-confirmed that levels of eNO are elevated in patients with SAR and do not differ significantly from those observed in patients with SAR+A. These findings are consistent with previously published data (20).

The production of NO is generally increased during inflammatory diseases, including asthma. The eventual fate of NO is oxidation to nitrite (NO_2) and nitrate (NO_3), the latter of which is less reactive than

other NO species (16). Increased levels of nitrate have previously been reported in EBC of asthmatics (21). In the present study, nitrate/nitrite levels were elevated in both groups of patients with rhinitis (SAR and SAR+A) compared to controls, and remained high when measured out of pollen season in both groups of patients. This finding is consistent with a long-lasting lower airway inflammation even in asymptomatic patients with SAR.

Increased exhaled CO observed in asthmatics and in patients with allergic rhinitis may be increased due to airway inflammation and subsequent induction of heme-oxygenase in the airway (22). In contrast with some other studies (23), we found no changes in exhaled CO levels in either patient group compared to healthy controls. Likewise, the baseline levels of exhaled CO in atopic asthmatics were not higher than those observed in control individuals; moreover, airway inflammation induced by whole lung allergen challenge did not cause any change in exhaled CO (17).

Finally, we show that nasal steroids could decrease oxidative stress markers since they significantly decrease levels of LTB₄ in EBC, with additional significant reduction of iso-8 in both groups of patients with SAR, but not inflammation, as reflected by increased eNO and nitrates in EBC. Similarly, the elevated levels of Cys-LTs in EBC of subjects with allergic rhinitis with/without concomitant asthma, were significantly suppressed after a 2-week intranasal treatment with 100 µg fluticasone propionate per nostril, once daily (24). Furthermore, treatment with nasal triamcinolone reduced hydrogen peroxide in the EBC of patients with allergic rhinitis (25).

In conclusion, our study shows that natural allergen exposure induces OS and airway inflammation in patients with seasonal allergic rhinitis even without any clinical symptoms of lower airway involvement. Besides, our study gives evidence that breath analysis with simultaneous evaluation of multiple markers in exhaled air and in EBC might be a useful non-invasive research tool to further understand the pathogenesis and progress of allergic airway diseases, as well as their response to treatment. However, the small sample size and the fact that only mild asthmatics were recruited in this study must be taken under consideration in the

interpretation of our findings.

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