NOVEL PERSPECTIVES IN THE DETECTION OF ORAL AND NASAL OXIDATIVE STRESS AND INFLAMMATION IN PEDIATRIC UNITED AIRWAY DISEASES

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United airway disease (UAD) concept proposed that asthma and rhinitis are both different clinical manifestation of a single inflammatory process. The aim of this study is to assess in upper and lower airways the level of inflammation and oxidative stress and to investigate the relationship between biomarkers in persistent allergic rhinitis (PER) and in concomitant asthma with PER. By a cross-sectional study we measured oral and nasal (FE_{NO}) and oral and nasal EBC 8-isoprostane, LTB_4 and PGE_2 in children with PER (n=14) and with PER and concomitant intermittent asthma (IA; n=25), mild persistent asthma (mA; n=28), moderate persistent asthma (MA; n=13) and in Healthy Controls (HCs; n=13). Oral and nasal FE_{NO} concentrations were increased in children with PER, IA, mA and MA when compared with HCs. Nasal 8-isoprostane was higher in EBC of children with PER and asthma than in HCs. Oral and nasal LTB_4 were higher in EBC of children with PER and mA than in HCs. Oral and nasal PGE_2 concentrations were higher in EBC of children with PER than in HCs. Positive correlations between oral and nasal biomarkers were found in IA for LTB4 and PGE2, in mA for FE_{NO}, 8-isoprostane, LTB_4 and PGE_2, and in MA for PGE_2. No correlations were observed in children with PER and HCs. Our results suggest that non-invasive markers of inflammation and oxidative stress might be useful to study the relationships between oral and nasal compartments in allergic children with PER and concomitant asthma with the aim of defining the UAD.

Asthma and allergic rhinitis (AR) are diseases characterized by the presence of chronic inflammation and oxidative stress. Concomitant nasal and bronchial inflammation in atopic asthma and AR (1-2) lead to the hypothesis of the so-called united airway disease (UAD) in patients with allergic airway disease (3). On the basis of this concept, it is important to detect and to monitor the presence of inflammation and oxidative stress in the upper and lower airways in patients with UAD both in the

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presence and in the absence of symptoms. This goal may be achieved by the use of non-invasive methods and this approach might be useful also to characterize different inflammatory phenotypes of patients and to monitor their disease in order to better "personalize" the therapy. To date, only few studies have addressed the comparative use of non-invasive methods, including bronchial and nasal fractional exhaled nitric oxide (FE_{NO}) and exhaled breath condensate (EBC), in patients with UAD (3).

FE_{NO} has been extensively studied as a potential non-invasive marker of asthma control and airway inflammation (1, 4) but, although the presence of correlation between FE_{NO} levels and clinical assessment (5) and between FE_{NO} and markers of oxidative stress, including 8-isoprostane and nitrotyrosine in EBC (6) have been demonstrated, the results supporting the routine use of these methodologies are still contradictory.

A large number of molecules measured in EBC, including leukotriene B_4 (LTB_4), Prostaglandin E_2 (PGE_2) and 8-isoprostane, are considered a useful markers of airway inflammation and oxidative stress in the airways of asthmatic children (7-9). LTB_4 is a pivotal biomolecule in the complex network of inflammatory mediators that characterizes allergic airway diseases; it is functionally involved in the neutrophilic inflammation of severe asthma and asthma exacerbations. Therefore, there are conflicting results on the role of LTB4 and 8-isoprostane since these markers seem to be relatively resistant to inhaled corticosteroids in allergic inflammation (9-11), although today 8-isoprostanes appear to be reliable biomarkers of oxidative stress during allergic airway inflammation (12). The relevant contribution of PGE_2 in asthma pathogenesis has been demonstrated by its contribution to the increase of eosinophil survival (13).

The aim of this study is to detect and to monitor the inflammation and the oxidative stress in the upper and lower airways of patients with atopic disease of UAD. This might allow to better understand the links between upper and lower airways and to have an insight in the assessment of the disease severity. In order to achieve this, we performed a cross-sectional study in atopic children with persistent AR (PER) and with different levels of asthma severity. For this objective we measured the oral and the nasal $FE_{NO,}$ and the concentrations of 8-isoprostane, LTB_4 and PGE_2 in oral and nasal EBC of pediatric patients since the use of non-invasive techniques are simple and easy to apply in children with atopic airway disease.

MATERIALS AND METHODS

Study design

The cross-sectional study included allergic children monosensitized to house dust mites with PER with or without concomitant asthma. All of them were evaluated by performing skin prick test (SPT), spirometry, oral and nasal FE_{NO}, oral and nasal EBC collection and symptom score evaluation. Patients were studied in a stable ambient condition to avoid the influence on the reliability of the above-mentioned procedures by temperature, barometric pressure and humidity. The study was approved by the local Ethics Committee, and written informed consent was obtained from the parents of the patients enrolled in the study.

Subjects

Sixty-six children with PER and with concomitant controlled asthma (31 males and 35 females), 14 children with PER without asthma (8 males and 6 females), and 13 age-matched healthy controls (7 males and 6 females) (HCs), which had negative SPT, were consecutively enrolled. Children were recruited among outpatients attending the Pulmonology/Allergy Department of the Italian National Research Council in Palermo. The study subjects had no acute respiratory symptoms during the previous four weeks.

Assessment of PER and asthma were based on current ARIA and GINA guidelines, respectively (14-15). PER diagnosis was performed at the study entry according to the history of nasal symptoms and positive SPT. Among the asthmatics, 25 children had intermittent asthma (IA) (treated with short-acting β_2-agonists on demand during the previous 3 months), 28 had mild persistent asthma (mA) (treated with 100 μg of fluticasone bid during the previous two months), and 13 children had moderate persistent asthma (MA) (treated with 250 μg of fluticasone propionate plus salmeterol 50 μg bid during the previous two months). All asthmatic children had AR and concluded the treatment for rhinitis (antihistamines or topical steroid treatment) 2 months before entering the study.

No patients had acute or chronic respiratory infections or exacerbation of asthma in the previous month or anatomical nasal disorders (i.e. nasal polyposis). Within one day from the EBC collection, all subjects performed

pulmonary function tests according to the international guidelines (16).

Assessment of atopic status

All subjects included in the study were assessed for atopic status which was established by clinical history and confirmed by SPT. Skin prick testing was performed as previously described (17). House dust mite (*Dermatophagoides pteronyssinus* and *Dermotophagoides farinae*) monosensitized patients were included in the study to select a population as homogeneous as possible and to avoid a bias caused by other allergen exposure. Serum samples were collected, and total serum IgE and specific IgE levels were determined by CAP System (Pharmacia-Upjohn, Uppsala, Sweden).

Spirometry

Baseline (forced expiratory volume in one second) FEV1 and forced vital capacity (FVC) were measured according to GINA guidelines (15). Baseline FEV1 was defined as the best of three reproducible curves. Patients were characterized by a reversible airway obstruction assessed by an increase of 15% of FEV_1 after inhalation of 200 μg of salbutamol.

Exhaled nitric oxide measurement

Oral and nasal FE_{NO} were determined by chemiluminescence (NIOX, Aerocrine, Sweden) following ATS/ERS recommendations (18). To evaluate the nasal FE_{NO}, the nasal samples were obtained from 1 nostril. Patients were instructed to blow up the cheeks to elevate the mouth pressure to isolate the nasal cavity from the rest of the respiratory system (1). The reproducibility of oral and nasal FE_{NO} with these methods was previously assessed (1).

EBC collection

Oral EBC was collected using a commercially available condenser (EcoScreen; Jaeger, Wurzburg, Germany). Children were asked to breathe at a normal frequency and tidal volume, wearing a nose clip, for 15 minutes. A median volume of 3 ml (2.6-3.5, percentiles) of oral EBC was collected.

Nasal EBC was collected according to Profita et al. (1). After applying a nasal mask to the EcoScreen condenser, children were asked to breathe through their noses, with their mouths closed. Before collection, the subjects were asked to rinse out their mouths. An aliquot of at least 1 ml of nasal condensate was collected and stored at -80°C in disposable polypropylene sterile tubes until analysed. A median volume of 2.3 ml (1.4-2.7, percentiles) of nasal EBC was collected. The reproducibility of oral and nasal EBC biomarkers with these methods was previously assessed (1).

Alfa-amylase activity was determined to exclude salivary contamination as previously described (20). None of EBC samples contained detectable levels of amylase activity.

EBC measurements

Concentrations of 8-isoprostane were measured with a specific RIA developed as previously described (9). LTB_4 and PGE_2 (Cayman Chemical, Milan, Italy) were assessed in EBC using commercially available specific EIA kits (10, 20). The detection limits for 8-isoprostane was 10 pg/ml. The limits of detection were less than 6 pg/ml and 40 pg/ml for LTB_4 and PGE_2 respectively. The reproducibility of EBC measurements was previously assessed (1). The intra-assay and inter-assay coefficients of variation for prostanoid RIAs were as follows: 8-isoprostane, < 2% and < 3%, respectively; PGE_2 < 4% and < 5%, respectively. Intraclass correlation coefficient for 8-isoprostane and PGE_2 were 0.95 and 0.90, respectively (21). The intra-assay and inter-assay coefficients of variations for LTB_4 were < 10% and < 15%, respectively (22). The intraclass correlation coefficient for LTB_4 was 0.72 (22).

Data analysis

Descriptive statistics were performed and quantitative data are expressed as median and percentiles (25 to 75 %). Comparison of quantitative variables between the different groups was made by non-parametric Mann Whitney test. The correlations between variables were performed using Spearman test correlation. The P-value less than 0.05 were considered statistically significant for both non-parametric Mann Whitney test and Spearman test correlation. StatView statistical software (SAS Institute, Cary, NC, USA) was used.

RESULTS

Demographic, clinical and atopic characteristics of the subjects

Demographic, clinical and atopic characteristics of the subjects enrolled in the study are shown in Table I.

Oral and nasal FE_{NO}

The levels of oral and nasal FE_{NO} were significantly higher in PER and in all asthmatic children than in HCs (Fig. 1A and B). No differences were observed among allergic children (Fig. 1A and B) (Table II).

Measurement of 8-isoprostane in EBC

No significant differences were observed in 8-

Table I. *Demographic, clinical and atopic characteristic of the subjects.*

Characteristic	HCs	PER	PER + Asthma			P value									
			IA	mA	MA	HCs v PAR	HCs v IA	HCs v mA	HCs v MA	PAR v IA	PAR v mA	PAR v MA	IA v mA	IA v MA	mA v MA
Subject number	13	14	25	28	13										
Male/Female	7/6	8/6	12/13	12/16	7/6										
Age (years)	10 (9-12.3)	12.5 (10-15)	9.0 (8.0-12.3)	11.0 (9.0-11.0)	10.0 (8.0-13.0)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FEV_1 (% predicted)	111.4 (103-114)	97.9 (98.5-115)	97.1 (88.6-99.5)	95.9 (91-98.6)	80.9 (75.6-83.3)	<0.0004	<0.0003	<0.0003	<0.0002	NS	NS	<0.0002	NS	<0.0002	< 0.00001
Total IgE (KU/ml)	89 (78-102.5)	318 (172-460)	388.5 (278.5-697)	516 (230-1067)	685 (270-1612)	<0.009	< 0.00001	< 0.00001	< 0.00001	NS	NS	NS	NS	NS	NS
Blood Eosinophil number (mm^3)	36.0 (34-51.5)	553 (263-600)	414 (322-665)	380 (204-497.5)	528 (394-706.5)	< 0.00001	< 0.00001	< 0.00001	< 0.00001	NS	NS	NS	NS	NS	<0.03
Disease duration (years)	none	4 (3-6)	5.8 (2.1-6)	5 (2.2-5)	5 (2.1-4.7)					NS	NS	NS	NS	NS	NS
Exacerbation number*	none	none	2 (0.5-2.8)	3.8 (0.9-6)	4 (1.5-7)								<0.04	<0.03	NS
RAST to mite (kU/L)	none	24.3 (11.1-37)	34.0 (13.9-47)	38.9 (19-40.9)	40.2 (19.9-50)					NS	NS	NS	NS	NS	NS

Results were expressed as median (25-75 percentiles)
Statistical analysis was performed by non-parametric Mann Whitney test
**Number of exacerbation during 12 months*
Abbreviations: PER = perennial allergic rhinitis; FEV_1 = forced expiratory volume in one second; HCs = healthy controls; IA = intermittent asthma; mA = mild persistent asthma; MA = moderate persistent asthma

Table II. *Levels of FE_{NO}, 8-Isoprostane, LTB_4 and PGE_2 in Bronchial and Nasal EBC in allergic children.*

Subjects	FE_{NO} (ppb)		8-Isoprostane (pg/ml)		LTB_4 (pg/ml)		PGE_2 (pg/ml)	
	Bronchial	Nasal	Bronchial	Nasal	Bronchial	Nasal	Bronchial	Nasal
HC	11.4 (6.1-15.1)	18.0 (14.8-31.0)	18.0 (16.0-30.0)	14.0 (10.0-18.5)	10.4 (0-54.8)	18.5 (9.8-33.8)	145.0 (106.7-159.9)	176.1 (115.3-264.7)
PER	25.0 (13.1-38.1)	37.5.2 (33.0-46.0)	18.0 (15.3-30.0)	23.0 (19.0-32.0)	31.4 (11.9-132.1)	82.5 (24.0-186.0)	29.0.5 (18.2-51.0)	30.7.0 (17.0.6-67.2)
PER+IA	26.0 (14-54.6)	41.0 (29.5.6-48.3)	26.0 (18.8-31.8)	26.0 (18.0-33.0)	26.0 (6.5-38.0)	27.4 (7.8-72.8)	121.3 (59.3-161.5)	181.5 (50.9-378.3)
PER+mA	21.8 (11.2-44.0)	34.5.2 (23.5.3-44.0)	23.5 (20.5-28.5)	22.5 (15.5-30.0)	44.0 (17.4-85.2)	25.2 (20.5-58.6)	73.8 (0-205.9)	76.6 (21.8-203.2)
PER+MA	38.1 (20.2-56.3)	41.0 (33.0.3-49.8)	24.0 (19.8-34.0)	21.0 (18.8-24.5)	15.5 (0-38.0)	3.2 (0-29.6)	45.0 (4.9-139.9)	27.4 (0-108.9)

Results were expressed as median (25-75 percentiles)

isoprostane levels in oral EBC among the study (Fig. 1C) while 8-isoprostane concentrations in nasal EBC were higher in PER (P =0.007), IA (P =0.0012), mA (P = 0.008), and MA (P =0.006) than in HCs. No differences were observed among allergic patients (Fig. 1D) (Table II).

LTB_4 measurements in EBC

LTB_4 concentrations in oral EBC were significantly higher in children with PER (P=0.04)

Table III. *Correlations between bronchial and nasal biomarkers in EBC.*

Markers	PER	PER+IA	PER + mA	PER + MA
FeNO (ppb)	No correlations	No correlations	Rho=0.5 P=0.01	No correlations
8-Isoprostane (pg/ml)	No correlations	No correlations	Rho=0.5 P=0.01	No correlations
LTB_4 (pg/ml)	No correlations	Rho=0.6 P=0.007	Rho=0.5 P=0.03	No correlations
PGE_2 (pg/ml)	No correlations	Rho=0.8 P=0.0004	Rho=0.5 P=0.01	Rho=0.8 P=0.009

Statistical analysis was performed by Spearman test

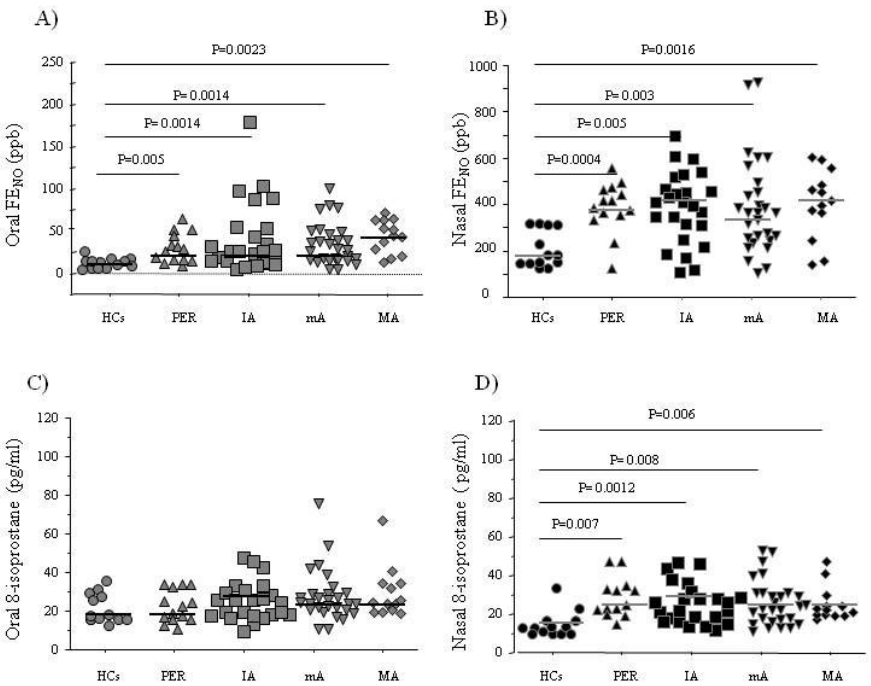

Fig. 1. *FE_{NO} and 8-isoprostane concentrations in 13 HC children, 14 children with PER and 62 children with asthma (25 children with IA, 28 children with mA and 13 children with MA, respectively). Panel **A)** concentrations of oral FE_{NO}. Panel **B)** concentrations of nasal FE_{NO}. Panel **C)** concentrations of 8-isoprostane in oral EBC. Panel **D)** concentrations of 8-isoprostane in nasal EBC. Data are shown as individual data. Statistical analysis was performed by Kruskall-Wallis test and* Dunn procedure. **p <0.05. Horizontal bars represent median values.*

and mA (P=0.012) than in HC subjects. LTB_4 concentrations in oral EBC were also higher in children with PER (P=0.04) and mA (P=0.018) than in children with MA (Fig. 2A). The levels of LTB_4 in nasal EBC were higher in children with PER (P=0.03) and mA (P=0.0064) than in HCs. Concentrations of nasal LTB_4 were higher in PER (P=0.0064), IA (P=0.05) and mA children (P =0.01)

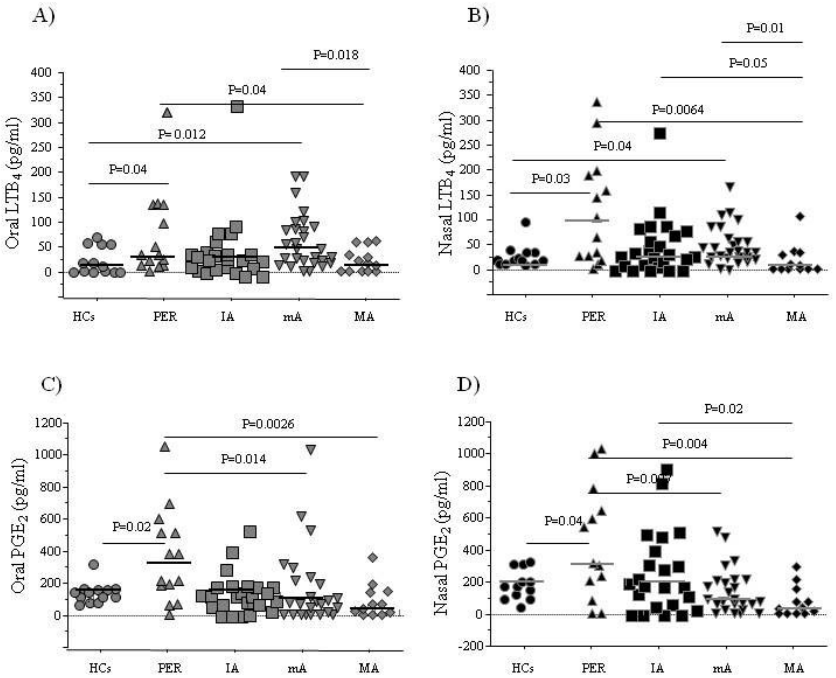

Fig. 2. *EBC LTB_4 and PGE_2 values in 13 HC children, 14 children with PER and 62 children with concomitant PER and asthma (25 children with IA, 28 children with mA and 13 children with MA, respectively). Panel **A**) concentrations of LTB_4 in oral EBC. Panel **B**) concentrations of LTB_4 in nasal EBC. Panel **C**) concentrations of PGE_2 in oral EBC. Panel **D**) concentrations of PGE_2 in nasal EBC. Data are shown as individual data. Statistical analysis was performed by Kruskall-Wallis test* and Dunn procedure. ** $P < 0.05$. Horizontal bars represent median values.*

than in MA children (Fig. 2B) (Table II).

PGE_2 measurements in EBC

The levels of PGE_2 in EBC were significantly higher in PER than in HCs (P =0.02), while lower levels were observed in MA (P=0.0026) and mA (P = 0.014) than in PER. The concentrations of PGE_2 in nasal EBC were higher in PER than in HC subjects (P =0.04). PGE_2 concentrations were higher in children with PER than in children with mA (P=0.007) and MA (P=0.004). Children with IA had higher PGE_2 concentrations than children with MA (P =0.02) (Fig. 2D) (Table II).

Correlations between bronchial and nasal FE_{NO}, 8-isoprostane, LTB_4, and PGE_2

In children with IA, positive correlations between oral and nasal EBC concentrations of LTB_4 (P=0.007, r=0.6) and between oral and nasal EBC concentrations of PGE_2 (P =0.0004, r =0.8) were observed (Table III).

In children with mA, there was a correlation between bronchial and nasal FE_{NO} concentrations (P=0.011, r =0.5), and between oral and nasal EBC concentrations of 8-isoprostane (P =0.01, r =0.5), LTB_4 (P =0.03, r = 0.5), and PGE_2 (P=0.01, r =0.05) (Table III).

In patients with MA, a correlation between oral and nasal EBC PGE_2 (P 0.009, r =0.8) was observed (Table III).

No correlations between markers of bronchial and nasal oxidative stress and inflammation were observed in children with PER and in HCs (Table III).

DISCUSSION

The present study investigates the levels of FE_{NO}, LTB_4, PGE_2 and 8-isoprostane in both upper and lower airway compartments of children with PER and with PER and different levels of asthma demonstrating that the presence of these markers is relevant in both districts, despite the big variability and the marginal differences in the groups. The

correlations between these markers and their persistence, despite children with PER and asthma being treated for asthma, might be able to mirror a relationship between biomarkers in the two airway compartments that might be useful to monitor and to phenotype UAD.

The high levels of FE_{NO} in both nasal and oral compartments of MA suggest that the oxidative stress associated with FE_{NO} might not be well controlled by recommended doses of inhaled corticosteroids (ICS). The presence of a positive correlation between the levels of oral and nasal FE_{NO} in children with mA is a further confirmation of the UAD concept, also in these patients with low levels of asthma severity. In addition, the fact that this correlation is not present in children with PER, IA and MA is consistent with a lower inflammatory burden in PER and IA. These results could be explained by the effects of ICS promoting a modification of FE_{NO} concentrations in both bronchial and nasal compartments of children with MA. On the other hand the issue that the assessment of relationship between bronchial and nasal levels of FE_{NO} is able to provide information about the effect of the treatment on inflammation in asthmatic children is consistent with the observation that in children with mA, treated with lower levels of ICS than MA patients, the correlations between the oral and nasal levels of FE_{NO} are persistent despite symptomatic and functional control of the disease.

The positive correlation between the levels of nasal and oral EBC 8-isoprostane in mA children with PER suggest that the concomitant nasobronchial evaluation of 8-isoprostane, together with the detection of the nasobronchial FE_{NO}, may be useful to biochemically phenotype this group of asthmatic children. Finally, there was no correlation between nasal and oral EBC 8-isoprostane in MA children with PER who received higher doses of ICS, suggesting that ICS might affect the interaction of nasal and bronchial inflammation.

We observed higher levels of nasal and oral EBC LTB_4 in mA children than in HCs, suggesting that the nasal-oral LTB_4-mediated inflammation persists despite treatment with ICS (9). Finally, we found a positive correlation between nasal and oral EBC LTB_4 concentrations in children with both IA and mA, suggesting that only treatment of both areas, the nose and bronchi, may lead, in these specific inflammatory phenotypes, to a complete control of upper and lower airway inflammation.

We showed higher levels of oral EBC and nasal PGE_2 concentrations in children with PER, indicating lower airway inflammation also in the absence of asthma symptoms. The presence of a positive correlation between nasal and oral EBC concentrations of PGE_2 in children with IA, mA and MA, is in agreement with the concept of UAD and with its relevant contribution in the pathogenesis of asthma. In addition, the findings that children with stable MA had lower LTB_4 and PGE_2 concentrations in oral and nasal EBC suggest that elevated doses of ICS might control both bronchial and nasal inflammation, likely through the involvement of humoral mechanisms involved in the interaction of the upper and lower airways in UAD (23). These results support the concept that the treatment of asthma may have a beneficial effect on the concomitant rhinitis (24-25).

The strength of this paper is to identify correlations between nasal and oral inflammation and oxidative stress in children with concomitant PER and different degrees of asthma defining "one-way/one-disease". The persistence of some correlations might identify inflammatory phenotypes of children with allergic airway disease and might help to plan an individualized therapy for UAD management. Nevertheless, the strong message of this paper needs to be further investigated opening new perspectives for larger and longitudinal studies aimed at assessing the concomitant evaluation of naso-bronchial markers of oxidative stress and inflammation to better manage children with UAD.

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