Inflammatory Markers and Acid-Base Equilibrium in Exhaled Breath Condensate of Stable and Unstable Asthma Patients

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Key Words

Asthma • Exhaled breath condensate • Oxidative stress • pH levels • Nitrite/nitrate • Ammonia

Abstract

Background: Nitrosative and acid stress play an important role in the pathogenesis of asthma. The aim of this study was to evaluate whether, in asthmatics, a link exists between the concentrations of nitrite/nitrate, ammonia and pH values in exhaled breath condensate (EBC) and asthma severity, lung function, exhaled nitric oxide (F_{ENO}), total IgE, eosinophil cationic protein (ECP) and blood eosinophilia. **Methods:** The above-mentioned parameters were measured in 19 healthy volunteers and 91 allergic asthmatics divided into three groups, i.e. 22 subjects with steroid-naïve stable asthma, 35 with inhaled corticosteroid (ICS)-treated stable asthma and 34 with ICS-treated unstable asthma. **Results:** Compared with healthy subjects, EBC from asthmatics had significantly lower pH values and ammonia concentrations and significantly higher levels of nitrite/nitrate. The extent of these changes was higher in patients with unstable than in patients with steroid-naïve and stable ICS-treated asthma. The

EBC pH was positively correlated with ammonia and negatively correlated with nitrite/nitrate, F_{ENO} or blood eosinophilia in all three groups of asthmatics. Significant positive correlations between EBC nitrite/nitrate and blood eosinophilia, ECP levels or F_{ENO} were observed in all groups of asthmatics. Significant negative correlations between EBC ammonia and nitrite/nitrate, F_{ENO} , ECP concentrations or blood eosinophilia were demonstrated in the groups of ICS-naïve and ICS-treated stable asthmatics. **Conclusions:** In asthmatic patients there is a relationship between EBC pH, ammonia and nitrite/nitrate concentrations and other recognized markers of airway inflammation. EBC pH values, ammonia and nitrite/nitrate levels measured together may help to assess airway inflammatory status and asthma severity.

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Introduction

Asthma is a condition involving chronic airway inflammation [1]. There is increasing evidence that chronic airway inflammation is associated with oxidative stress manifested by elevated levels of oxidative products such

as reactive oxygen [e.g. hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-)] and nitrogen species [e.g. nitric oxide (NO), nitrite (NO_2^-), nitrate (NO_3^-) and peroxynitrite (ONOO^-)] [2–4]. In asthmatic airways activated inflammatory cells produce large amounts of NO, which easily penetrates the cell membranes and can be found in exhaled air [5]. NO is highly reactive in solution and is rapidly converted to nitrite and nitrate [6], which can be detected in exhaled breath condensate (EBC). In addition to oxidative stress, acid stress can also play a role in the pathogenesis of asthma [7]. Acidification of the airway lining fluid can affect airway function through numerous mechanisms, including bronchoconstriction [7], impaired ciliary motility [8], increased viscosity of airway mucus [9] and damage to the airway epithelium [10]. These changes could all be crucial for the development of asthma [7–10]. Hunt et al. [11] have hypothesized that the chronic airway acidification observed in asthma could be neutralized by ammonia produced from glutamine by the airway epithelium. This is based on the observation that during acute asthma exacerbation EBC ammonia concentrations are low when pH is low [11]. The relevance of ammonia in asthma pathophysiology is also supported by the observation that both EBC ammonia and pH are negatively correlated with exhaled nitric oxide (F_{ENO}), a sensitive marker of asthmatic airway inflammation [12]. These findings suggest that the airway acid-base equilibrium may be closely related to the concentration of NO metabolites (nitrite, nitrate) [12].

Airway inflammation in asthma can be evaluated indirectly by testing lung function, eosinophil counts in induced sputum [13] or the level of F_{ENO} [14], or directly by bronchoscopy with bronchoalveolar lavage [15] and biopsy [16]. In recent years attention has focused on EBC as a noninvasive method of investigating the lungs [17]. EBC can be collected by cooling the exhaled air during spontaneous breathing [6]. Molecules present in the EBC are derived from bronchial and/or alveolar aerosols and from evaporation of the airway lining fluid [6]. The principal component of EBC is condensed water vapor, which contains volatile and nonvolatile components including a large number of mediators [18]. Concentrations of these mediators are thought to be influenced by airway inflammation and are associated with lung diseases, especially asthma [6].

Consequently, the aims of this study were to assess markers of nitrosative and acid stress (pH, nitrite/nitrate and ammonia), reflecting airway inflammation in the EBC of patients with asthma and to determine the potential relationship between these biomarkers and asthma

severity, lung function or other indices of airway inflammation [F_{ENO} , total IgE, eosinophil cationic protein (ECP) or blood eosinophilia].

Patients and Methods

Patients

Ninety-one asthmatics were enrolled in the study as follows: 22 subjects with steroid-naïve, mild allergic asthma (21 women, 1 man; mean age 29 years; range 19–52 years), 35 subjects with stable, mild-to-moderate, inhaled corticosteroid (ICS)-treated allergic asthma (20 women, 15 men; mean age 42 years; range 18–73 years) and 34 subjects with severe, unstable, ICS-treated allergic asthma (10 women, 24 men; mean age 49 years; range 25–74 years). Asthma was diagnosed according to the criteria recommended by GINA 2002 [19]. Patients with asthma exacerbation, which is defined below, were excluded from the study. Patients with any other respiratory disease or any concomitant malignant, heart, renal, liver or collagen disease were also excluded.

Asthma exacerbation was defined as an intensification of clinical symptoms, a decrease in spirometric values and an increase in the consumption of rescue medications. These changes required the intensification of anti-inflammatory treatment (increase in inhaled steroid dose and/or addition of oral corticosteroids) and, if respiratory tract infection was confirmed, therapy with antibiotics. The infection was ruled out based on the patient's history and clinical examination as well as WBC measurement. Patients who had had respiratory tract infections in the month prior to the study were excluded from this program.

The steroid-naïve asthmatics had not been treated with ICS during the previous 3 months. The majority of patients in that group were subjects with recently diagnosed, never-treated asthma. They were free from acute exacerbations and respiratory tract infections during the 3 months prior to the study. Patients with stable, mild-to-moderate asthma had been treated with low-to-medium doses of ICS at a constant dose for at least 3 months. Stable asthma was defined as a minimal need for rescue medications (short-acting β_2 -agonists), no exacerbations and no use of systemic steroids in the previous 12 months. The patients with severe, unstable asthma had required one or more hospitalizations for asthma and more than three oral steroid bursts in the previous year. They had been taking high doses of ICS and long-acting β_2 -agonists for at least 6 months. All patients were atopic and sensitized to common inhaled allergens, as evaluated by skin prick tests.

A total of 19 healthy subjects (14 women, 5 men; mean age 32 years; range 19–51 years) participated in the study as a control group. They were free of respiratory tract infection within the 3 months prior to the study and from other significant illnesses known to affect F_{ENO} measurements. Asthma patients and healthy volunteers were nonsmokers who during the previous year had not been passive smokers.

All of the patients and healthy volunteers were examined by a physician and then underwent EBC collection, F_{ENO} measurement and spirometry. Blood samples were collected to determine serum total IgE, ECP and blood eosinophil count.

The study protocol was approved by the Ethics of Research Committee of the Medical University of Białystok (number of agreement: R-I-002/139/2009). Informed consent was obtained from each patient.

Measurements

F_{ENO} was measured by chemiluminescence technique using a Sievers 280i NO Analyzer (Boulder, Colo., USA). The measurements were performed at an expiratory flow of 50 ml/s according to ATS recommendations for online measurement of F_{ENO} in adults [20].

Spirometry (forced expiratory volume in 1 s, FEV_1) was performed using a MasterScreen Pneumo PC spirometer (Jaeger, Höchberg, Germany) according to ATS standards [21].

EBC was collected by using a commercially available condenser (EcoScreen I; Jaeger) according to the current ATS/ERS guidelines [20]. All measurements were performed at the same time (8.00–10.00 a.m.) to avoid possible circadian rhythm of mediator concentrations in the EBC. All patients were asked to refrain from eating and drinking before the collection of EBC. Exhaled air entered and left the chamber through one-way valves and the inlet and outlet, thus keeping the chamber closed. A low temperature inside the condensing chamber throughout the collection time produced a cooling-down sample. The temperature of collection was around 0°C [20, 22]. Patients were instructed to breathe tidally for 10 min with a nose clip. The respiratory rate ranged from 15 to 20 breaths/min. Patients were asked to swallow their saliva periodically and to temporarily discontinue collection if they needed to cough. At the end of collection 1.5- to 3.5-ml aliquots of condensate were transferred to Eppendorf tubes, immediately frozen and thawed shortly before use. Samples were stored at –80°C for no longer than 3 months [23]. They were frosted and defrosted only once. Throughout this study the nonconsumable parts of the condenser were extensively washed with ammonia-free water.

pH values in the EBC were assessed after deaeration by bubbling with argon as previously described [24, 25], using a pH-meter with microelectrode (Elmetron, Zabrze, Poland). Stable pH was achieved in all cases after deaeration of the condensate with argon (350 ml/min) for 10 min. The EBC pH was measured at 22°C.

Ammonia in the EBC was determined by the spectrophotometric method described by Chaney and Marbach [26]. Briefly, 0.5 ml phenol reagent (0.5 M phenol and 0.01 M sodium nitroprusside) and hypochlorite reagent (0.625 M NaOH and 0.03 M sodium hypochlorite) were added successively to 100 µl of EBC and after 30 min of color development at 40°C the absorbance was measured on a spectrophotometer (Unicam, Leeds, UK) at 625 nm. The other constituents present in the EBC did not interfere with the color development. Detection limit of this method was 10 µM.

The total NO_2^-/NO_3^- level in the EBC was measured by a two-step procedure. The NO_3^- was first reduced to NO_2^- and then the total NO_2^- (converted NO_3^- plus NO_2^-) was detected by colorimetric Griess reaction. Conversion of NO_3^- to NO_2^- was performed by enzymatic reduction using NADPH-dependent nitrate reductase (from *Aspergillus* spp.; Boehringer Mannheim, Germany) as described by Schmidt and Kelm [27]. Briefly, aliquots of EBC were supplemented with NADPH, FAD and nitrate reductase to yield final concentrations of 50 µM, 5 µM and 200 U/l, respectively, and were incubated for 30 min at 37°C. To remove the unreacted

NADPH, samples were further incubated for 5 min with lactate dehydrogenase (from rabbit muscle; Boehringer Mannheim) and sodium pyruvate added to the final concentrations of 10 mg/ml and 10 mM, respectively. The samples were then mixed with an equal volume of Griess reagent and after 10 min of color development at room temperature the absorbance was measured at a wavelength of 540 nm. Values obtained by this procedure represent the sum of nitrite and nitrate. Detection limit in this study was 0.2 µM, which was comparable to that reported by other authors [28].

Serum total IgE concentrations and serum ECP were measured using ImmunoCAP™ Technology (Pharmacia Diagnostics, Uppsala, Sweden). The minimum detectable level was 2.0 µg/l. Blood eosinophil count was measured using a hematologic analyzer (Coulter Electronics GmbH, Miami, Fla., USA).

Analyses

Statistical analyses were completed using the Mann-Whitney U test. All values were expressed as means $\pm$ SD; $p < 0.05$ were considered significant. Correlations were evaluated by Spearman's rank test.

Results

The demographic and clinical characteristics as well as the mean values of studied parameters in all three groups of asthmatic patients and healthy volunteers are summarized in table 1.

Differences in studied parameters between particular groups of asthmatics and healthy volunteers are presented in table 2.

pH Values

Figure 1 shows that in asthmatic patients a wide range of EBC pH values (4.75–7.91) can be observed. As is shown, compared with healthy subjects the mean pH values were significantly lower in all groups of asthmatic patients (healthy subjects: 7.58 ± 0.21 ; steroid-naïve asthmatics: 7.29 ± 0.49 , $p = 0.04$; ICS-treated stable asthmatics: 7.32 ± 0.48 , $p = 0.02$, and ICS-treated unstable asthmatics: 7.1 ± 0.51 , $p < 0.001$). The lowest mean pH value was observed in the group of unstable asthmatics treated with ICS. Their mean pH value differed significantly ($p = 0.04$) from that in the group of stable asthmatics treated with ICS.

As shown in table 3, the pH values of EBC in asthmatic patients correlated positively with ammonia levels and negatively with nitrite/nitrate, F_{ENO} and blood eosinophil counts. There was no correlation between the pH value of EBC and spirometric indices (FEV_1) in patients with asthma. In the groups of steroid-naïve and ICS-treated stable asthmatics a negative correlation between EBC pH and ECP levels was observed. In the group of patients

Table 1. Characteristics of studied asthmatic patients and healthy volunteers

Characteristics	Healthy volunteers	Stable asthma steroid-naïve	Stable asthma ICS-treated	Unstable asthma ICS-treated
Number of patients	19	22	35	34
Sex (F/M)	14/5	21/1	20/15	10/24
Age, years	32.00 ± 10.12	29.50 ± 8.37	42.42 ± 12.26	49.82 ± 10.70
Duration of symptoms, years		2.31 ± 1.04 ^{c, d}	9.14 ± 5.15 ^{b, d}	14.41 ± 6.60 ^{b, c}
pH of EBC	7.58 ± 0.21	7.29 ± 0.49 ^a	7.32 ± 0.48 ^{a, d}	7.1 ± 0.51 ^{a, c}
EBC nitrite/nitrate, μM	9.02 ± 3.33	12.46 ± 5.34 ^a	13.56 ± 5.88 ^a	15.45 ± 7.91 ^a
EBC ammonia, μM	436.10 ± 11.60	358.00 ± 173.67 ^{a, d}	366.40 ± 111.83 ^{a, d}	278.04 ± 97.27 ^{a-c}
Baseline FEV ₁ , % predicted	103.82 ± 9.63	91.80 ± 10.80 ^{a, d}	85.97 ± 21.07 ^{a, d}	52.31 ± 14.86 ^{a-c}
Blood eosinophil count, cells/mm ³	54.15 ± 22.01	201.09 ± 80.30 ^{a, d}	239.82 ± 85.51 ^{a, d}	289.70 ± 87.09 ^{a-c}
Serum concentration of ECP, μg/l	4.02 ± 0.84	13.72 ± 4.54 ^{a, c, d}	10.62 ± 4.09 ^{a, b, d}	19.06 ± 7.80 ^{a-c}
Serum total IgE concentration, kU/l	59.94 ± 25.14	236.72 ± 166.38 ^{a, d}	200.85 ± 94.84 ^{a, d}	267.52 ± 100.05 ^{a-c}
F _{ENO} , ppb	13.57 ± 4.08	64.55 ± 54.19 ^a	39.05 ± 19.39 ^{a, d}	51.14 ± 23.06 ^{a, c}

Data are presented as means ± SD. ^a p < 0.05 vs. healthy volunteers; ^b p < 0.05 vs. patients with steroid-naïve asthma; ^c p < 0.05 vs. patients with stable ICS-treated asthma, and ^d p < 0.05 vs. patients with unstable ICS-treated asthma.

Table 2. Differences in studied parameters between particular groups of asthmatics and healthy volunteers (p values)

	Healthy volunteers vs. ICS-naïve	Healthy volunteers vs. ICS-stable	Healthy volunteers vs. ICS-unstable	ICS-naïve vs. ICS-stable	ICS-naïve vs. unstable	ICS-stable vs. unstable
pH	0.04*	0.02*	<0.001*	0.85	0.06	0.04*
Ammonia	0.045*	0.04*	0.01*	0.24	0.043*	0.045*
Nitrite/nitrate	0.04*	0.003*	<0.001*	0.56	0.27	0.31
FEV ₁	0.01*	<0.001*	<0.001*	0.29	<0.001*	<0.001*
F _{ENO}	<0.001*	<0.001*	<0.001*	0.37	0.99	0.02*
IgE	<0.001*	<0.001*	<0.001*	0.48	0.03*	0.008*
EOS	<0.001*	<0.001*	<0.001*	0.09	<0.001*	0.02*
ECP	<0.001*	<0.001*	<0.001*	0.01*	0.004*	<0.001*

EOS = Eosinophilia. * Indicates significant difference.

with unstable ICS-treated asthma the pH value negatively correlated with total IgE concentrations.

Ammonia

Figure 2 shows that the mean ammonia levels in EBC were significantly lower in all groups of asthmatics compared with healthy subjects (healthy subjects: 436.10 ± 11.60 μM; steroid-naïve asthmatics: 358.00 ± 173.67, p = 0.045; ICS-treated stable asthmatics: 366.40 ± 111.83, p = 0.04, and ICS-treated unstable asthmatics: 278.04 ± 97.27, p = 0.01). The lowest mean ammonia level was observed in the group of unstable asthmatics treated with ICS. Their mean ammonia levels differed significantly (p = 0.04) from those in the group of stable asthmatics

treated with ICS. As far as differences in mean EBC ammonia concentrations between the studied groups of asthmatics are concerned, a significant difference (p < 0.045) was observed between patients with steroid-naïve and ICS-treated stable or unstable asthma.

As shown in table 4, the EBC ammonia levels in asthmatic patients positively correlated with the EBC pH. Correlations between the EBC ammonia levels and FEV₁ or total IgE were not found. In the groups of ICS-naïve and ICS-treated stable asthma, a negative correlation between EBC ammonia and nitrite/nitrate, F_{ENO}, ECP concentrations or blood eosinophilia was shown. There were no correlations between EBC ammonia levels and FEV₁ or total IgE in all studied asthmatic patients.

Fig. 1. pH levels in EBC of the three groups of asthmatic patients and the group of healthy volunteers. Horizontal bars represent median values.

Fig. 2. Ammonia levels in EBC of the three groups of asthmatic patients and the group of healthy volunteers. Horizontal bars represent median values.

Table 3. Correlations (r values) between pH of EBC and other studied parameters in the group of healthy subjects and the three groups of asthmatics

	Healthy volunteers	ICS-naïve asthma	Stable asthma ICS-treated	Unstable asthma ICS-treated
EBC ammonia	0.109	0.70*	0.74*	0.42*
EBC nitrite/nitrate	-0.056	-0.83*	-0.78*	-0.77*
FEV ₁	-0.50*	-0.05	0.07	0.09
F _{ENO}	0.088	-0.90*	-0.87*	-0.72*
IgE	0.46*	0.10	-0.18	0.56*
EOS	-0.174	-0.53*	-0.91*	-0.39*
ECP	-0.519*	-0.79*	-0.92*	-0.31

EOS = Eosinophilia. * p < 0.05.

Table 4. Correlations (r values) between concentration of ammonia in EBC and other studied parameters in the group of healthy subjects and the three groups of asthmatics

	Healthy volunteers	ICS-naïve asthma	Stable asthma ICS-treated	Unstable asthma ICS-treated
EBC pH	0.109	0.70*	0.74*	0.42*
EBC nitrite/nitrate	-0.50*	-0.74*	-0.62*	-0.33
FEV ₁	0.42*	0.07	-0.04	0.16
F _{ENO}	-0.17	-0.68*	-0.69*	-0.30
IgE	0.36	-0.01	-0.13	0.09
EOS	-0.27	-0.46*	-0.65*	-0.09
ECP	-0.05	-0.58*	-0.64*	-0.12

EOS = Eosinophilia. * p < 0.05.

Nitrite/Nitrate

Figure 3 shows that the mean concentrations of nitrite/nitrate in the EBC were significantly higher in all groups of asthmatics compared with healthy controls (healthy subjects: $9.02 \pm 3.33 \mu\text{M}$; steroid-naïve asthmatics: 12.46 ± 5.34 , $p = 0.04$; ICS-treated stable asthmatics: 13.56 ± 5.88 , $p = 0.003$, and ICS-treated unstable asthmatics: 15.45 ± 7.91 , $p < 0.001$).

As shown in table 5, the EBC nitrite/nitrate concentrations in asthmatic patients negatively correlated with pH and positively correlated with F_{ENO} or blood eosinophilia or ECP levels. There were no correlations between nitrite/nitrate levels in EBC and spirometric indices (FEV₁). In the groups of ICS-naïve and stable ICS-treated asthmatics, a negative correlation between nitrite/nitrate and ammonia in EBC was shown. NO₂/NO₃ levels in EBC were

Fig. 3. Nitrite/nitrate levels in EBC of the three groups of asthmatic patients and the group of healthy volunteers. Horizontal bars represent median values.

Table 5. Correlations (*r* values) between concentration of nitrites/nitrates in EBC and other studied parameters in the group of healthy subjects and the three groups of asthmatics

	Healthy volunteers	ICS-naïve asthma	Stable asthma ICS-treated	Unstable asthma ICS-treated
EBC pH	-0.05	-0.83*	-0.78*	-0.77*
EBC ammonia	-0.50*	-0.74*	-0.62*	-0.33
FEV ₁	-0.28	0.07	-0.08	0.19
F _{ENO}	0.57*	0.85*	0.80*	0.82*
IgE	0.01	-0.01	0.02	-0.45*
EOS	0.27	0.59*	0.78*	0.37*
ECP	0.27	0.70*	0.75*	0.36*

EOS = Eosinophilia. * *p* < 0.05.

negatively correlated with total IgE only in the group of patients with unstable ICS-treated asthma.

In each group of asthmatics we assessed the number of patients who had low pH, low ammonia and high nitrite/nitrate in EBC simultaneously. Low and high values were defined, taking into account the mean values obtained in healthy controls (EBC pH < 7.58, EBC ammonia < 436.10 µM and EBC nitrite/nitrate > 9.02 µM). It was found that in the group of patients with steroid-naïve asthma there were 12 subjects with abnormal pH, ammonia and nitrite/nitrate levels (54%), in the group of pa-

tients with ICS-treated stable asthma there were 15 subjects (54%) and in the group of patients with ICS-treated unstable asthma there were 22 subjects (64%). Taking into account all patients with asthma, low pH and ammonia and high nitrite/nitrate levels in the EBC were observed simultaneously in 53 patients (58%).

Discussion

In our study we showed that EBC pH values in asthmatic patients were significantly lower compared with healthy subjects. These results confirm and extend the data that Kostikas et al. [25] have found in asthmatic adults and Carraro et al. [12] have observed in asthmatic children. Moreover, taking all studied groups of asthmatics into account, we noted the lowest pH values in patients with unstable asthma. This observation can be interpreted to mean that endogenous airway acidification could rise when asthma severity increases.

Kostikas et al. [25] have shown a negative correlation between EBC pH levels and eosinophil numbers in the induced sputum of asthmatic patients. It has been hypothesized that the granule matrix of eosinophils contains eosinophil peroxidase, an enzyme that, in the presence of H₂O₂, is able to oxidize halides to highly reactive hypohalous acids [29]. This might in part explain the low pH that was observed in stable patients with moderate asthma, characterized by higher eosinophil counts and H₂O₂ values than those with mild asthma [29]. The above-mentioned hypothesis is confirmed in our study, since we showed that EBC pH values negatively correlate with peripheral blood eosinophilia in all studied groups of asthmatics and with concentrations of ECP (a marker of eosinophil activation) in patients with steroid-naïve and stable ICS-treated asthma. To our knowledge, our study is the first in which pH, ammonia, nitrite/nitrate in EBC and peripheral blood eosinophilia and ECP were measured together in asthmatic patients with different degrees of severity of the disease. We also found (data not shown here and not published so far) that in patients with unstable asthma the levels of H₂O₂ in EBC were significantly higher than in controls.

Hunt et al. [11] demonstrated that cultured epithelial cells from the airway and lung may produce ammonia stoichiometrically from glutamine in a reaction catalyzed by glutaminase. It is hypothesized that the production of ammonia represents an attempt by epithelial cells to buffer the acidic challenge, thus maintaining the airway pH homeostasis [12]. The authors reported that the

activity of glutaminase is downregulated by inflammatory cytokines such as interferon- γ and tumor necrosis factor- α [11], and upregulated by corticosteroid administration, which suppresses inflammatory cytokines [11, 30]. In addition, they observed that during acute asthma exacerbations both EBC ammonia levels and pH values were low, while glutaminase expression was decreased, suggesting that in asthmatic subjects the inhibition of glutaminase may contribute to lowering the airway pH [11]. This observation was further confirmed by the study performed by Carraro et al. [12], which showed that ammonia levels in EBC are lower in asthmatic children compared with controls and that there is a positive correlation between ammonia concentrations and pH in EBC. Therefore, our findings are in agreement with these observations, since we found that in the three studied groups of asthmatics, ammonia levels in EBC were decreased compared with healthy volunteers and the pH of EBC was positively correlated with EBC ammonia levels.

The involvement of ammonia in asthma pathophysiology is supported by the study of Carraro et al. [12], who have shown that both EBC ammonia and pH are negatively correlated with F_{ENO} , a sensitive marker of asthmatic airway inflammation. These correlations let the authors speculate that the airway acid-base equilibrium may affect the concentration of nitrite/nitrate [12]. This is consistent with the observation of Hunt et al. [11] who showed that airway acidity favors the protonation of nitrite, which is increased in asthma [31], to form unstable nitrous acid (HNO_2), which can be subsequently decomposed to produce NO [12]. In our study the pH of EBC from asthmatic patients negatively correlated both with nitrite/nitrate concentrations and the level of F_{ENO} . Here we report a statistically significant negative correlation between ammonia concentrations in EBC and F_{ENO} in the groups of patients with steroid-naïve and ICS-treated stable asthma but not in those with unstable disease. Higher ammonia in EBC in patients with unstable ICS-treated asthma may result from the upregulation of glutaminase expression by high doses of administered inhaled and systemic corticosteroids [30]. However, further studies are needed to confirm this hypothesis.

Nitrite, which is present in low micromolar concentrations in the airway lining fluid, is converted readily to NO in mildly acidic environments [24]. In all groups of asthmatic patients in our study the levels of nitrite/nitrate in EBC were strongly correlated, especially with F_{ENO} and with ECP and blood eosinophilia. This observation may suggest that the nitrite/nitrate concentration in EBC

could also reflect eosinophilic inflammation, which is a characteristic feature of asthma.

It has been reported that the acidification of the airways in asthma may produce many damaging effects [32]. Therefore, the pH value of EBC may reflect the airway acid-base equilibrium and inflammatory status. In fact, our findings show that EBC pH is negatively correlated with asthma severity. This observation has been confirmed in previous studies. Kostikas et al. [33] revealed that EBC pH is lower in patients with poorly controlled compared to controlled asthma. In line with this there are observations of Ratnawati et al. [34], suggesting that EBC pH may be an additional marker of asthma control in children. Moreover, this parameter is easy to measure in EBC and the time of storage (not exceeding 2 years) does not change the pH [35].

In a recently published study, Malinovschi et al. [36] revealed that EBC nitrates, but not nitrites, were related to asthma control. However, most studies report measurements of a combination of nitrates and nitrites [34, 37]. In our study the sum of NO_2/NO_3 in EBC was measured and this parameter was raised when asthma severity increased. It is still a matter of debate as to which of these NO metabolites measured in EBC is better related to airway inflammation [36]. Horváth et al. [18] have suggested that nitrites are unstable NO metabolites and the measurement of nitrite alone in EBC would probably be meaningless. More studies are needed, therefore, to define which parameter would be more suitable for assessing in EBC and which is more closely related to asthma control.

In each group of asthmatics more than half the patients had low pH, low ammonia and high nitrite/nitrate in EBC simultaneously. The percentage of such patients was highest in the group of unstable ICS-treated asthmatics. Therefore, our observations suggest that pH, ammonia and nitrite/nitrate, measured together in EBC, could be helpful in the assessment of asthma severity. However, further studies performed on larger groups of patients are needed to confirm this hypothesis.

There are some limitations to the study. One of them could be the predominance of women in the steroid-naïve group and the group of healthy volunteers, which is a consequence of the selection of patients for this study. Paget-Brown et al. [38], in a study performed on 404 healthy subjects, revealed that there were no differences in EBC pH based on sex. In our opinion the predominance of women in the two previously mentioned groups of patients has had no impact on the results of the study and their interpretation. Another weak point is the difference

in age in the studied groups, which is a consequence of the natural history of the disease. Asthma was diagnosed at a young age. Therefore, steroid-naïve asthmatics are younger than those with unstable disease. The patients with unstable disease are older because of the longer duration of symptoms of the disease (the duration of symptoms of asthma is presented in table 1). In fact, the results of experiments performed by Dragonieri et al. [39] indicate that the composition of EBC of young and older subjects differs. This suggests that the pH of their EBC may also differ. However, in studies published by Paget-Brown et al. [38] performed on 404 healthy subjects of all ages, by Vaughan et al. [35] performed on 76 subjects (range 18–48 years) and by Brooks et al. [40] (23 normal younger individuals, median age 24 years and 25 older participants, median age 72 years), subject age had no effect on EBC pH.

Conclusions

The results of our study suggest that there is a relationship between acid-base equilibrium and markers of nitrosative stress in EBC and other parameters commonly used in the assessment of inflammatory status in asthmatic patients (spirometry, F_{ENO} , ECP, blood eosinophilia and total IgE). All three parameters (pH, ammonia and nitrite/nitrate) measured together in EBC in asthmatics could be helpful in the assessment of airway inflammation and asthma severity.

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