

ORIGINAL ARTICLE

Urates in exhaled breath condensate as a biomarker of control in childhood asthmaMarta Navratil, PhD¹, Davor Plavec, PhD^{1,2}, Damir Erceg, PhD^{1,2}, Sandra Bulat Lokas, MSc¹, Jelena Živković, MSc¹, and Mirjana Turkalj, PhD^{1,2}¹Srebrnjak Children's Hospital, Reference Center for Clinical Pediatric Allergology of the Ministry of Health, Zagreb, Croatia, and ²Faculty of Medicine, Josip Juraj Strossmayer University Osijek Trg Svetog Trojstva 3, 31000 Osijek, Croatia**Abstract**

Objective: The aim of this study was to (1) investigate the possibility to use urates in exhaled breath condensate (EBC) as a biomarker of airway inflammation and control in childhood asthma and (2) explore their association with other biomarkers of airway inflammation and clinical indices of asthma control (Asthma Control Test [ACT], quality of life [PAQLQ], lung function, *prn* beta-agonist use, time from last exacerbation [TLE]). **Methods:** This cross-sectional study comprised 103 consecutive patients (age 6–18 years) divided in groups of uncontrolled (INC, *n* = 53) and controlled asthma (IC, *n* = 50). Measured lung function and biomarkers included: spirometry, eosinophilic cationic protein (ECP), high-sensitivity C-reactive protein (hs-CRP), exhaled NO (F_ENO), pH and urates in EBC and exhaled breath temperature (EBT). **Results:** Statistically significant differences were found between groups for EBC urates, EBC pH and EBT (NC versus C: EBC urates, median [IQR], μmol/L; 10 [6] versus 45 [29], *p* < 0.001; EBC pH, mean [SD], 7.2 [0.17] versus 7.33 [0.16], *p* = 0.002; EBT mean [SD], °C; 34.26 [0.83], versus 33.90 [0.60], *p* = 0.014). EBC urates showed significant association with TLE and F_ENO (*r* = 0.518, *p* < 0.001; *r* = 0.369, *p* = 0.007, respectively) in NC, and EBC pH (*r* = 0.351, *p* < 0.001), FEV₁ (*r* = 0.222, *p* = 0.024), ACT (*r* = 0.654, *p* < 0.001), PAQLQ (*r* = 0.686, *p* < 0.001) and *prn* salbutamol use (*r* = −0.527, *p* < 0.001) in all asthmatics. **Conclusion:** In our study, EBC urates were found to be the best single predictor of asthma control and underlying airway inflammation. Our results provide evidence supporting the potential utility to use EBC urates as an additional non-invasive biomarker of control in childhood asthma.

Introduction

Asthma is the most common chronic disease in childhood in developed countries [1] that is characterized by chronic inflammation of the airways involving variable and recurrent airflow obstruction and increased airway responsiveness to a variety of stimuli [2]. Airway inflammation is the main underlying cause of the recurrent episodes of airflow limitation in asthma [3] and is associated with increased oxidative and acid stress [4–6].

Over the last 15 years, there has been increasing interest in the non-invasive assessment of airway inflammation in addition to the traditional measures of asthma control and severity [7]. Although numerous biochemical and cellular biomarkers have been evaluated in previous studies, an ideal measure of asthma activity, underlying inflammation and control has not been identified [8].

Recent findings, measuring high-sensitivity C-reactive protein (hs-CRP), support a hypothesis of persistent systemic

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inflammation in asthma going on in parallel with local inflammation [9]. Endogenous airway acidification assessed by exhaled breath condensate (EBC) pH is strongly related to underlying inflammatory process in asthma and is affected by the degree of oxidative stress, and by end products of nitric-oxide (NO) metabolism [6]. Altered oxidant–antioxidant balance was also associated with airways obstruction in asthmatics [4], and urates emerged as one of the main antioxidants in nasal airway secretions [10]. Recently, investigators from our group reported EBC to contain a measurable urate concentration, significantly higher in children with controlled asthma in comparison to children during an acute exacerbation suggesting EBC urates as a new non-invasive biomarker in asthma management [11].

We hypothesized that urates in EBC could be used as a useful non-invasive biomarker of inflammation, oxidative stress and control of childhood asthma. The aim of this study was to (1) investigate the possibility to use urates in exhaled breath condensate (EBC) as a biomarker of airway inflammation and control in childhood asthma and (2) explore their association with other biomarkers of airway inflammation and clinical indices of asthma control (asthma control test [ACT], quality of life [PAQLQ], lung function, *prn* beta-agonist use and time from last exacerbation [TLE]).

Correspondence: Marta Navratil, MD, Reference Center for Clinical Pediatric Allergology of the Ministry of Health, Srebrnjak Children's Hospital, Srebrnjak 100, HR-10000 Zagreb, Croatia. Tel: 385-1-6391-143. Fax: 385-1-6319-188. E-mail: marta.navratil@gmail.com

Subjects and methods

Subjects

One-hundred and three consecutive children and adolescents with asthma aged 6–18 years, of which 25 girls (24.3%) were included in this study between June 2011 and December 2012 from our outpatient clinic. Asthma was diagnosed based on ATS and GINA guidelines [7,12] at least a year before the inclusion visit. Characteristics of studied subjects are shown in Table 1. At the time of evaluation, 82 (79.6%) children were, according to seasonality, exposed to aeroallergens to which they were sensitized. Patients were on the stable dosage of their regular asthma treatment, with exception of *prn* SABA (Table 1), during the time from preceding clinical visit (1–3 months ago, depending on patients' level of asthma control). Patients were not taking vitamin supplementation therapy, or various brands of N-acetyl cysteine, or other antioxidants during the preceding month. Diagnostic work-up was performed according to standardized in-house procedure (according to GINA guidelines), and in line with ethical principles (approved by Hospital Review Board) and Declaration on Human Rights from Helsinki 1975 and Tokyo amendments 2004–2008 [7,13]. All subjects and/or parents consented for the study.

Exclusion criteria included subjects with diabetes mellitus, cancer status, obesity (body mass index [BMI] over the 85th percentile for age [14]), systemic inflammatory disorders, subjects with serum CRP levels of >2.5 mg/L, subjects with a respiratory tract infection during the preceding month and subjects with a gastro-esophageal reflux disease. Gastro-esophageal reflux disease was excluded in controlled asthmatics by anamnestic and clinical data (characteristic signs and symptoms) or by 24 h pH monitoring study in uncontrolled asthmatics [15].

Asthma control was evaluated according to GINA guidelines [7] by two respiratory physicians with special interest in

asthma (M. N. and M. T.) who were blinded for the results of biomarker measurements. Patients were divided in two groups according to GINA guidelines [7]: controlled asthma (C), $n=50$, 48.5%) versus uncontrolled asthma (NC), $n=53$, 51.5%) based on daytime symptoms (more than twice a week), limitations of activities (any), nocturnal symptoms (any), need for reliever (more than twice a week), PEF measurement ($<80\%$ predicted or personal best) and exacerbations (one or more a year). The NC group included patients that would be, according to GINA guidelines, classified as partly controlled and uncontrolled.

Methods

Medical history, including asthma symptoms, adherence to asthma medication plan, *prn* use of SABA, health resource utilization, were assessed together with physical examination, lung function measurement, $F_{E}NO$ and EBT measurement. Blood sampling for hs-CRP, ECP, total and specific IgE were done upon clinical examination between 8.00 am and 12.00 am. Two validated questionnaires, the ACT and pediatric quality of life questionnaire (PAQLQ) were used to assess asthma control and quality of life.

Sensitization status was determined by skin prick tests (SPT) and/or increased level of specific IgE. The SPT were performed according to the EAACI guidelines [16], with 11 aeroallergens (Stallergenes, France): *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat dander, dog dander, moulds (*Alternaria alternata*, *Cladosporium herbarum*), hazel tree pollen (*Corylus avellana*), birch pollen (*Betula verrucosa*), grass pollen mixture (*Phleum pratense*, *Lolium perenne*, *Dactylis glomerata*, *Festuca elatior*, *Poa pratensis*), short ragweed pollen (*Ambrosia elatior*) and mugwort pollen (*Artemisia vulgaris*). Negative (saline solution) and positive (histamine 10 mg/mL) controls were used. The tests have been considered positive if a mean wheal diameter was ≥ 3 mm compared to a negative control.

Table 1. Characteristics of studied subjects ($N=103$).

	All ($N=103$)	NC ($n=53$)	C ($n=50$)
Gender, male, No (%)	78 (75.7)	39 (73.6)	39 (78)
Age, mean (SD) [range], year	11.7 (3.2) [6–18]	11.8 (3.5) [6–18]	11.6 (2.9) [6–18]
BMI, mean (SD), kg m^{-2}	18.7 (2.5)	18.4 (2.6)	18.9 (2.3)
Atopic status, No (%)			
No sensitization	18 (17.5)	11 (20.75)	7 (14)
Mono-sensitized	31 (30.1)	18 (33.96)	13 (26)
Poly-sensitized	54 (52.4)	24 (45.3)	30 (60)
Allergen exposure, No (%)	82 (79.6)	41 (77.36)	41 (82)
Total IgE, median (IQR), kU/L	249 (420)	407 (474)	226.5 (285)
Current asthma treatment, No (%)			
Only SABA <i>prn</i>	17 (16.5)	9 (16.9)	8 (16)
ICS	30 (29.1)	15 (20.7)	19 (38)
LTRA	7 (6.8)	4 (7.5)	3 (6)
ICS + LABA	42 (40.8)	25 (47.2)	17 (34)
ICS + LTRA	5 (4.8)	2 (3.8)	3 (6)
ICS + LABA + LTRA	2 (1.9)	2 (3.8)	0 (0)
SABA used ^a		42 (79.25)	5 (10)
Past exacerbation, No (%) ^b		43 (81.1)	0

SD, standard deviation; BMI, body mass index; IQR, interquartile range; *prn*, as needed; SABA, short acting beta-agonists; ICS, inhaled corticosteroids; LTRA, leukotrien antagonists; LABA, long acting beta-agonists; NC, uncontrolled asthma; C, controlled asthma.

^a $\chi^2=49.73$, $df=1$, $p<0.001$.

^b $p<0.001$ (Fisher exact test).

The concentrations of total and allergen-specific IgEs were determined by the fluorescence enzyme immunoassay (FEIA; ImmunoCAP, LKB, Uppsala, Sweden) method on a selective UniCAP 100 auto-analyzer (LKB, Uppsala, Sweden). The concentration of hs-CRP was determined within 1 h by latex-enhanced immunoturbidimetric method [17] on a Beckman Coulter AU 400 automated biochemistry analyzer (Beckman Coulter, Tokyo, Japan), using Beckman Coulter reagents (Beckman Coulter Life and Material Science Europe, Hamburg, Germany).

FE_{NO} was measured with the single exhalation method at 50 mL/s during 10 s using a NiOX analyzer (Aerocrine, Stockholm, Sweden) according to current ERS/ATS recommendations [18].

Lung function measurements (FEV₁, FEV₁/FVC, FVC, MEF₂₅, MEF₅₀, MEF₇₅, PEF, post-bronchodilator FEV₁, post-bronchodilator FEV₁/FVC, post-bronchodilator FVC) were done using a computerized pneumotach (Ganzhorn, Germany) in accordance with ATS guidelines [19] and presented as percentage of predicted values according to Quanjer et al. [20].

EBC samples were obtained between 7:00 and 9:00 am. Samples were collected according to the ATS/ERS task force recommendation [21] using an EcoScreen condenser (Erich Jaeger GmbH, Oechberg, Germany). Samples were deaerated (CO₂ elimination, i.e. gas standardization) using argon (350 mL/min for 10 min). None of the EBC samples showed detectable α -amylase catalytic activity (detection limit 7 U/L). Measurements of pH in EBC were performed up to 5 min after argon deaerating using a blood gas analyzer (Ecosys, Eschweiler, Germany). Urates measurements were performed up to 10 min after EBC collection by an enzymatic color test on a Beckman Coulter AU 400 selective auto-analyzer (Beckman Coulter, Tokyo, Japan). The detection limit for urates concentration was 5 μ mol/L (our validation data for analytical sensitivity).

The childhood ACT (C-ACT) and the ACT were used to assess asthma control [22,23]. C-ACT is a seven question survey (score range, 0–27) for children ages 4–11 years in which the questions are completed by both the patient and parent. ACT is a patient-completed five-question survey (score range, 5–25) designed for adults and adolescents 12 years or older. According to published reports and validation data, a C-ACT or ACT score of 19 or lower is defined as having uncontrolled asthma during the previous 4 weeks [24]. Questionnaires were filled in before patients performed any other procedures. For children 6–11 years old, C-ACT was completed by the children (four questions) and their parents (three questions). For children 12 years and older, all five questions were completed by the patients. The answers to each question were summed to obtain the total C-ACT or ACT score.

The quality of life was assessed by PAQLQ which consists of 32 questions in four domains: activity limitation, symptoms, emotional function and environmental stimuli. Responses in each domain and an overall score are graded on a seven-point scale, where 1 represents “total impairment” and 7 represents “no impairment”. The overall PAQLQ score is the mean of all 32 responses [25].

Statistical analysis

Data storage and processing for statistical analysis was performed using Microsoft Excel 2013 (Microsoft, Redmond, WA). Continuous variables were described as mean and standard deviation (SD) if they had normal distribution, or median and interquartile range (IQR) if not. BMI was adjusted for age using percentile categories [18]. Comparisons between groups were made using a Student's *t*-test for normally distributed variables or Mann–Whitney test for non-normally distributed and using χ^2 -test or Fisher exact test for categorical variables. Associations were analyzed using regression analysis (univariate and multivariate models). Age was always used as a covariate in the models. For categorical variables, logistic regression analysis was used. Discriminant analysis was used to depict variables that significantly discriminate between controlled and uncontrolled asthma. For the assessment of biomarkers as predictors of asthma control, receiver operating characteristic (ROC) curves were calculated. Areas under the ROC curves (AUC) with 95% confidence intervals (CI) and their differences from 0.5 were calculated. Sensitivities, specificities, positive (PPV) and negative (NPV) predictive values, positive (LR+) and negative (LR–) likelihood ratios were calculated for the optimal cut-points. The data were analyzed using STATISTICA version 10 (StatSoft, Inc. Tulsa, OK) and MedCalc version 12 (MedCalc Software, Mariakerke, Belgium). Statistical significance was set to $p < 0.05$ for all tests.

Results

Data on all outcomes for all 103 asthmatic patients is presented in Table 1. There were no statistically significant differences between groups for distribution of age, gender, atopic status, asthma therapy during the last 3 months and allergen exposure ($p > 0.05$ for all comparisons). Significant differences between groups were found for the need for rescue medication and for having an exacerbation during the last year ($p < 0.001$ for both comparisons; Table 1).

Comparisons between controlled and uncontrolled asthma

As expected, statistically significant differences between groups were found for spirometric parameters (FEV₁, FEV₁/FVC, MEF₅₀, PEF), C-ACT, ACT and PAQLQ ($p < 0.05$ for all; Tables 2 and 3).

The values of EBC urates, EBC pH and EBT were significantly different between studied groups (NC versus C: EBC urates, median [IQR], μ mol/L; 10 [6] versus 45 [29], $p < 0.001$; EBC pH, mean [SD], 7.2 [0.17] versus 7.33 [0.16], $p = 0.002$; EBT mean [SD], °C; 34.26 [0.83], versus 33.90 [0.60], $p = 0.014$; Table 4 and Figures 1–3). This was not the case for other biomarkers (FE_{NO}, hs-CRP and ECP; $p > 0.35$ for all; Table 4).

Discriminant analysis depicted EBC urates as the best single biomarker as a predictor of asthma control ($F = 62.969$, $p < 0.001$, diagnostic accuracy of 80.6%). Using ROC curve analysis (Figure 4), best cut-off value for EBC urates was 16 μ mol/L to differentiate between the two groups of

Table 2. Comparison of lung function parameters between controlled and uncontrolled asthma ($N = 103$).

	NC ($n = 53$)	C ($n = 50$)	Statistics ^a
FEV ₁ , median (IQR), % of predicted	92 (19)	96.5 (19)	$Z = -2.7021, p = 0.007$
FEV ₁ /FVC, median (IQR)	80 (12)	85 (9)	$Z = -2.5998, p = 0.009$
MEF ₅₀ , mean (SD), % of predicted	75.6 (23.4)	90.8 (19.7)	$t = -2.442, p < 0.001$
PEF, mean (SD), % of predicted	89.5 (17.2)	96.2 (16.1)	$t = -2.048, p = 0.043$

NC, uncontrolled asthma; C, controlled asthma; SD, standard deviation; IQR, interquartile range.

^aGroup comparisons were done using Student's t -test (t -value) or Mann-Whitney test (Z -value).Table 3. Comparison of ACT and quality of life scores between controlled and uncontrolled asthma ($N = 103$).

	NC ($N = 53$)	C ($N = 50$)	Statistics ^a
C-ACT, median (IQR), score	18 (3) ($n = 30$)	25 (3) ($n = 26$)	$Z = -5.670, p < 0.001$
ACT, median (IQR), score	18 (3) ($n = 23$)	25 (1) ($n = 24$)	$Z = -5.863, p < 0.001$
PAQLQ, median (IQR), score	5.53 (0.79)	7 (0.16)	$Z = -8.443, p < 0.001$

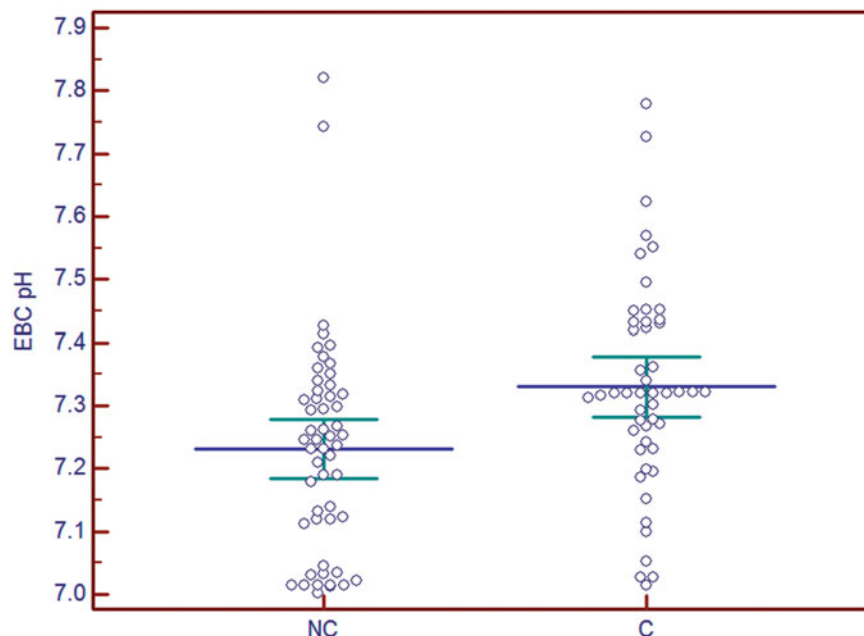
NC, uncontrolled asthma; C, controlled asthma; C-ACT, Childhood Asthma Control; IQR, interquartile range; PAQLQ, Pediatric Asthma Quality of Life Questionnaire Test; ACT, Asthma Control Test.

^aGroup comparisons were done using Mann-Whitney test (Z -value).Table 4. Comparison of biomarkers (BMs: F_ENO, EBC parameters, EBT, ECP and hs-CRP) between controlled and uncontrolled asthma ($N = 103$).

BMs	NC ($N = 53$)	C ($N = 50$)	Statistics ^a
F _E NO, median (IQR), ppb	22 (25)	21 (23)	$Z = 0.934, p = 0.3505$
EBC urates, median (IQR), $\mu\text{mol/L}$	10 (6)	45 (29)	$Z = -7.460, p < 0.0001$
EBC pH, mean (SD)	7.23 (0.17)	7.33 (0.16)	$t = -3.068, p = 0.0028$
EBT, mean (SD), $^{\circ}\text{C}$	34.26 (0.83)	33.9 (0.6)	$t = 2.495, p = 0.0142$
hs-CRP, median (IQR), mg/L	0.5 (0.78)	0.39 (0.73)	$Z = 0.419, p = 0.6752$
ECP, median (IQR), ng/mL	17.6 (11.4)	15.7 (16.8)	$Z = 0.917, p = 0.3590$

NC, uncontrolled asthma; C, controlled asthma; SD, standard deviation; IQR, interquartile range; F_ENO, exhaled NO; EBC, exhaled breath condensate; EBT, exhaled breath temperature; hs-CRP, high sensitivity CRP; ECP, eosinophilic cationic protein.^aGroup comparisons were done using Student's t -test (t -value) and Mann-Whitney test (Z -value).

Figure 1. EBC pH in uncontrolled (NC) and controlled (C) asthmatics. Heavy horizontal lines represent mean and whiskers represent 95% confidence intervals for means.



asthmatics with an AUC of 0.927 (95% CI 0.858–0.969), a sensitivity of 94.0%, specificity of 81.1%, PPV of 82.5% and NPV of 93.5%. EBC pH and EBT showed modest discriminative power for asthma control (EBC pH, $F = 9.412$,

$p = 0.003$, diagnostic accuracy 64.1%; EBT, $F = 6.225$, $p = 0.014$, diagnostic accuracy 60.2%).

Finally, asthma control was best described by the combination of biomarkers (ECP, EBT, EBC urates; $F = 43.174$,

Figure 2. EBC urates in uncontrolled (NC) and controlled (C) asthmatics. Heavy horizontal lines represent median and whiskers represent 95% confidence intervals for medians.

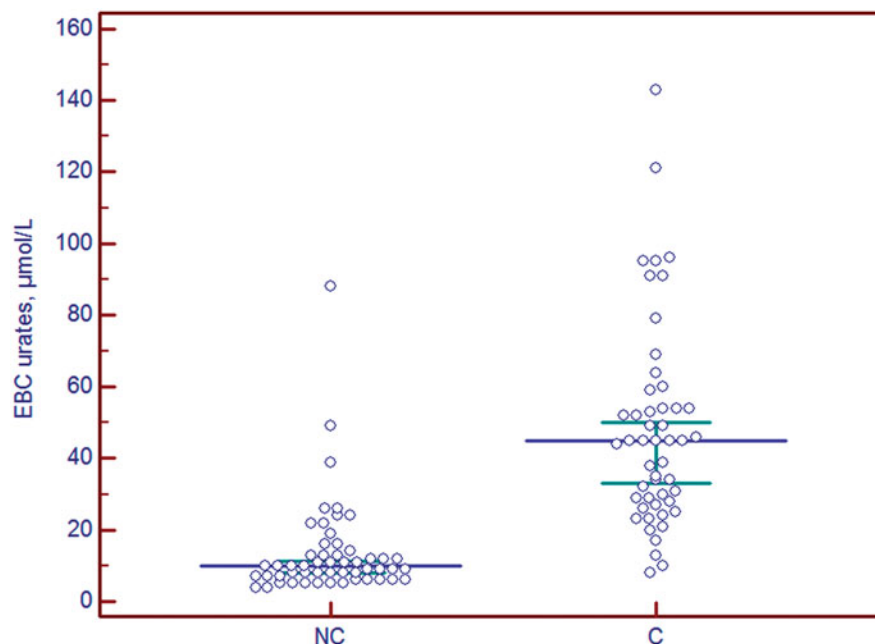
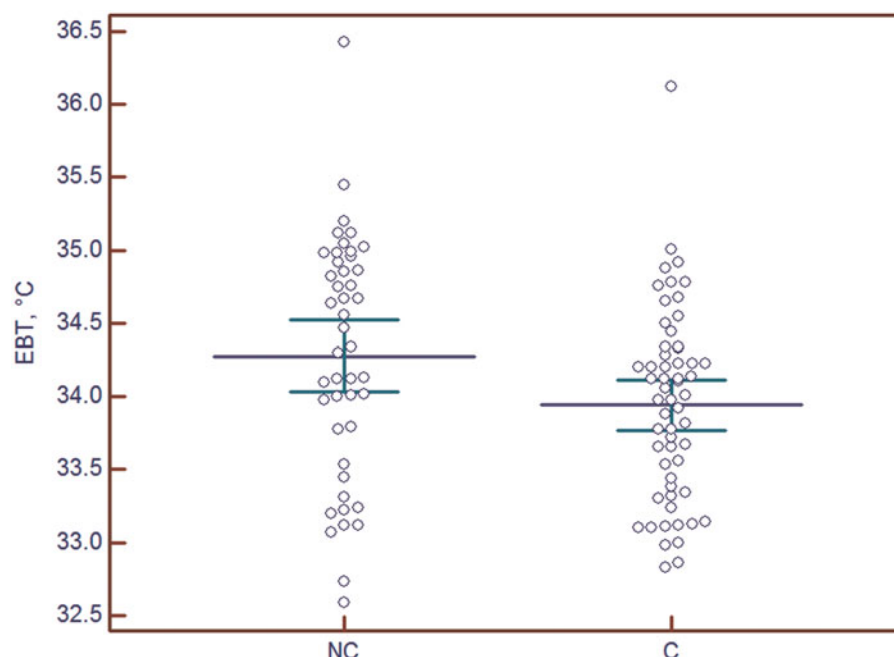


Figure 3. EBT in uncontrolled (NC) and controlled (C) asthmatics. Heavy horizontal lines represent mean and whiskers represent 95% confidence intervals for means.



$p < 0.001$, diagnostic accuracy 87.4%) although ROC curve analysis was not significantly different with AUC of 0.931 (95% CI, 0.863–0.971), sensitivity of 94%, specificity of 83.0%, PPV of 83.9% and NPV of 93.6% (Figure 5).

Associations of local and systemic biomarkers with parameters of asthma control (*prn* salbutamol use, ACT, PAQLQ, lung function and TLE) and in between each other

In the univariate regression analysis, serum ECP had a mild but significant positive correlation between EBC urates ($r = 0.223$, $p = 0.024$) and $F_{E}NO$ ($r = 0.379$, $p < 0.001$), and an inverse correlation with ACT ($r = -0.365$, $p = 0.007$). Serum hs-CRP had a mild significant positive correlation with

prn salbutamol use ($r = 0.240$, $p = 0.014$), EBT ($r = 0.445$, $p < 0.001$, Figure 6) and an inverse correlation with FEV_1 (% predicted; $r = -0.366$, $p = 0.007$), post-bronchodilator FEV_1/FVC ($r = -0.201$, $p = 0.044$), ACT ($r = -0.316$, $p = 0.021$) and PAQLQ ($r = -0.373$, $p = 0.006$).

EBC pH had a very mild but significant positive correlation with ACT ($r = 0.258$, $p = 0.009$), PAQLQ ($r = 0.287$, $p = 0.003$) and a significant inverse correlation with *prn* salbutamol use ($r = -0.259$, $p = 0.008$) and $F_{E}NO$ ($r = -0.296$, $p = 0.020$). EBC urates had a mild significant positive correlation with EBC pH ($r = 0.351$, $p < 0.001$), FEV_1 (% predicted) ($r = 0.222$, $p = 0.024$) and a moderate significant correlation with measures of asthma control and QoL, ACT ($r = 0.654$, $p < 0.001$, Figure 7) and PAQLQ ($r = 0.686$, $p < 0.001$, Figure 8), and a significant inverse

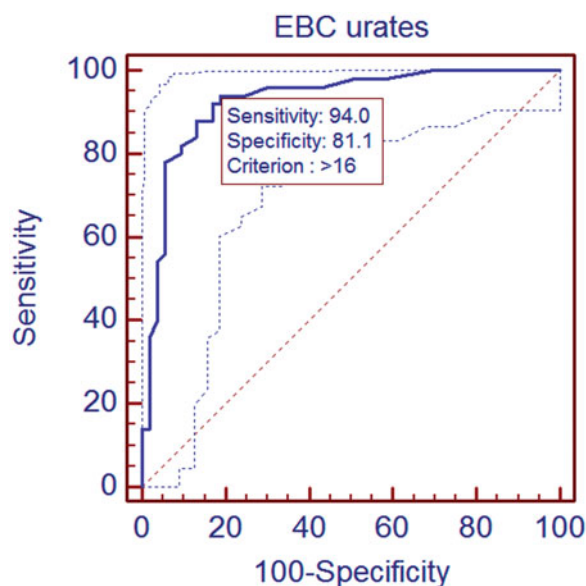


Figure 4. Receiver operating characteristics (ROC) curve to define the best cut-off of EBC urates to differentiate between uncontrolled and controlled asthmatics.

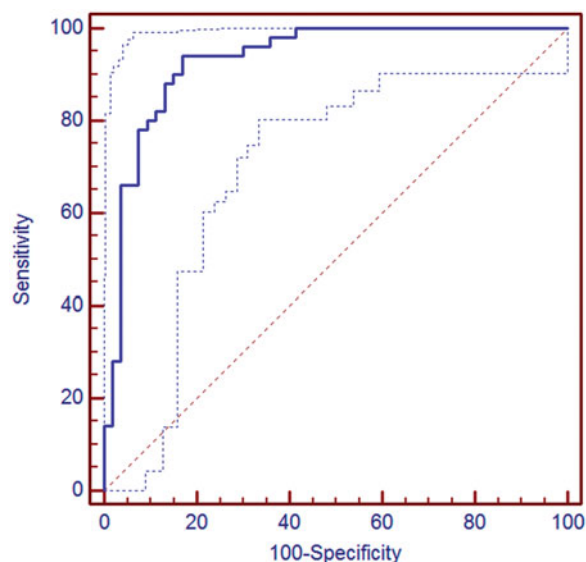


Figure 5. Receiver operating characteristics (ROC) curves for combination of BMs (ECP, EBT, EBC urates in the identification of uncontrolled asthma in the whole study population).

correlation with *prn* salbutamol use ($r = -0.527$, $p < 0.001$). In a group of uncontrolled asthma, EBC urates were moderately significantly positively correlated with TLE ($r = 0.572$, $p < 0.001$, Figure 9) and mildly with $F_{E}NO$ ($r = 0.369$, $p = 0.007$). EBT had a very mild but significant positive correlation with *prn* salbutamol use ($r = 0.293$, $p = 0.003$), and an inverse mild correlation with ACT ($r = -0.365$, $p = 0.007$) and PAQLQ ($r = -0.372$, $p = 0.006$) in all asthmatics.

Discussion

In this study, we found that children with uncontrolled asthma had increased local airway inflammation, increased acid and oxidative stress compared to children with controlled asthma.

Uncontrolled asthma was thus characterized by increased EBT, decreased EBC pH and decreased concentration of EBC urates, respectively. EBC urates were the best single biomarker of underlying airway inflammation predicting asthma control. EBC urates were significantly mildly to moderately associated with traditional measures of asthma control; FEV_1 , *prn* salbutamol use and with ACT and PAQLQ scores.

Purinergic signaling regulates airway defense mechanisms and has been implicated in lung injury and in the pathogenesis of a wide range of respiratory disorders and diseases, including asthma [26]. Extracellular adelyn purines are elevated in EBC in patients with asthma and cystic fibrosis and proposed as biomarkers of neutrophilic airways inflammation [27,28]. Uric acid is a product of the metabolic breakdown of purine nucleotides. At physiologic pH, uric acid is present as urates. Although plasma urates are secreted to RELF together with mucus, there is evidence that respiratory tract epithelial cells may also be involved in the synthesis of urates by purine metabolism [10]. There is increasing evidence showing urates as a strong antioxidant *in vivo* and a novel oxidative stress marker [29].

Decreased EBC urates in uncontrolled, compared to controlled asthma, could be explained as a consequence of oxidant/antioxidant imbalance. On the other hand, the puzzling observation from a previous study that EBC urates in controlled asthmatics show significantly higher concentrations than in healthy subjects could be explained by the elevated antioxidative potential in asthma or an effect of anti-inflammatory treatment [11]. However, EBC is a diluted “body fluid” which consists of water vapor and aerosol [21]. It is greatly diluted than RELF, but exact degree of dilution is still unknown. According to Effros, dilution of non-volatile compounds is 1:12 000 and could be influenced by disease state [30,31]. It is possible that airway inflammation, acid and oxidative stress in uncontrolled asthma interfere with micro aerosol generation, resulting in reduced concentrations of non-volatile biomarkers in EBC even if airway concentrations are unchanged (or increased). It seems plausible, and perhaps likely, that the decreased EBC urates concentrations in severe asthma reflect disease mediated changes in dilution of airway secretions in EBC and not changes of uric acid concentrations on airway surfaces.

According to our results, EBC urates could be a useful biomarker of controlled asthma having a high NPV of 93.5% at a cut-off level of $>16 \mu\text{mol/L}$. However, in an everyday practice, it would be more appropriate to have a biomarker of underlying inflammation in uncontrolled asthma, since inaccurate perception of symptoms is often found in asthmatics [32]. Changing the cut-off level for EBC urates to $<27 \mu\text{mol/L}$, we can have a NPV of 92.9% and PPV of 82% for the uncontrolled asthma. Adding to these observations, an inverse correlation between EBC urates and TLE in our study additionally supports the possible consumption of urates during asthma exacerbations. Furthermore, we found a correlation of lung function measurements with EBC urates what is in agreement with work of Ueno et al. [33].

We also found a decreased EBC pH in uncontrolled asthmatics being in agreement with previous reports by Kostikas et al. [6] in adults and Carraro et al. [34] in children. These results could be explained by a raised endogenous

Figure 6. Relationship between serum high-sensitivity C-reactive protein (hs-CRP) and exhaled breath temperature (EBT; $r = 0.445$, $p < 0.0001$).

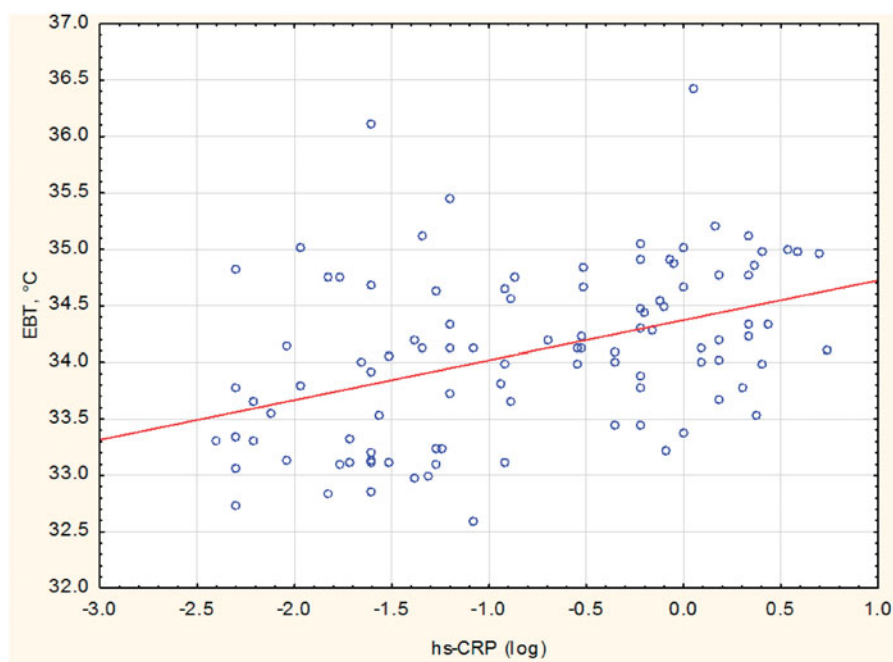
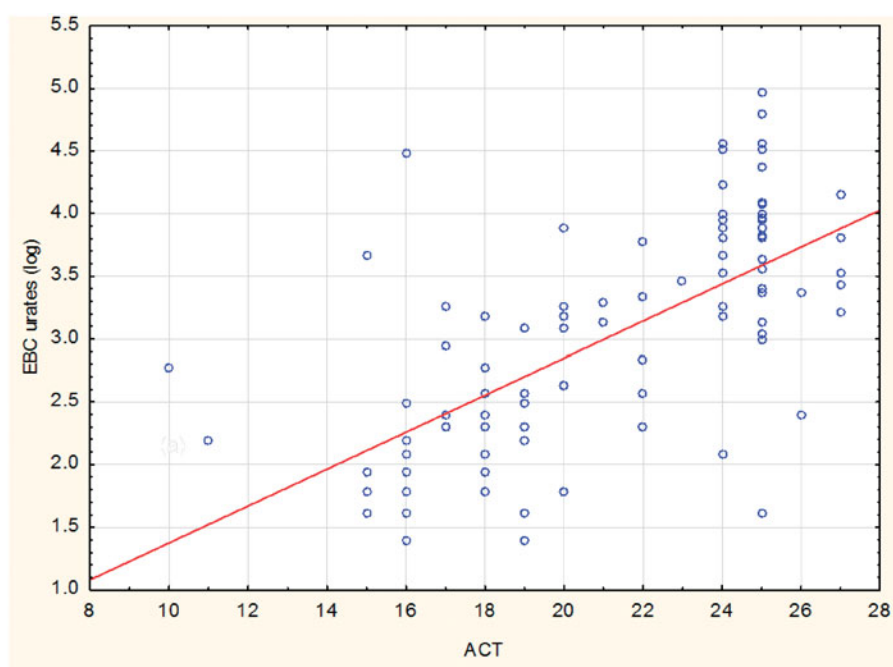


Figure 7. Relationship between exhaled breath condensate EBC urates and asthma control test (ACT; $r = 0.654$, $p < 0.0001$).



airway acidification when asthma control was lost. Kostikas et al. [6] also reported a significant association between EBC pH and sputum eosinophilia, total nitrate/nitrite and markers of oxidative stress referring an asthma pathomechanism of initial cellular process leading to oxidative stress, NO production and finally, acid stress. A significant correlation between EBC hydrogen peroxide (H_2O_2) level and nitrite/nitrate level in asthmatics was also found in a work of Ueno et al. [33]. In agreement with their results, our results concerning EBC urates showed a positive correlation with $F_{E}NO$ level in uncontrolled asthmatics, supporting an association of eosinophilic airway inflammation and NO metabolism with oxidative stress.

In addition, we have observed an association of oxidative stress and acid stress (assessed by EBC urates and EBC pH, respectively) what is somewhat opposite to the results of Zhao et al. [35]. We can hypothesize that, in childhood asthma, these two stresses are parallel and complementary to each other although we cannot explain the difference between our findings and findings of Zhao et al. except by a different pathophysiological patterns in uncontrolled asthma in children and adults. Kostikas et al. also found significant association of acid and oxidative stress in steroid-treated patients with asthma [6], although they assessed oxidative stress using a different marker than in the work of Zhao et al. [35].

Figure 8. Relationship between serum exhaled breath condensate (EBC) urates and pediatric asthma quality of life questionnaire test (PAQLQ; $r = 0.686$, $p < 0.0001$).

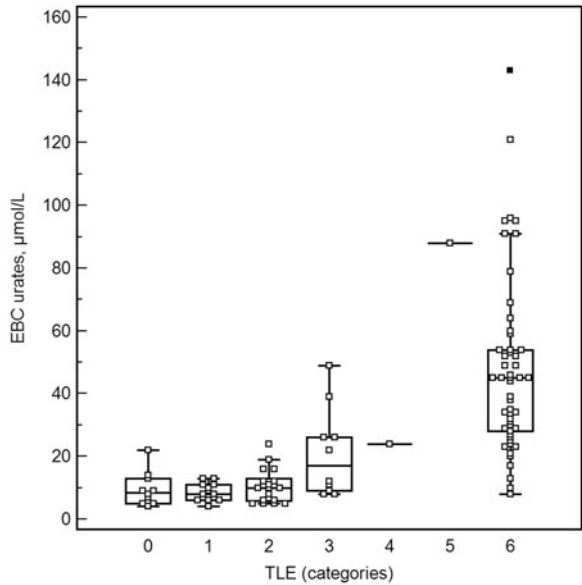
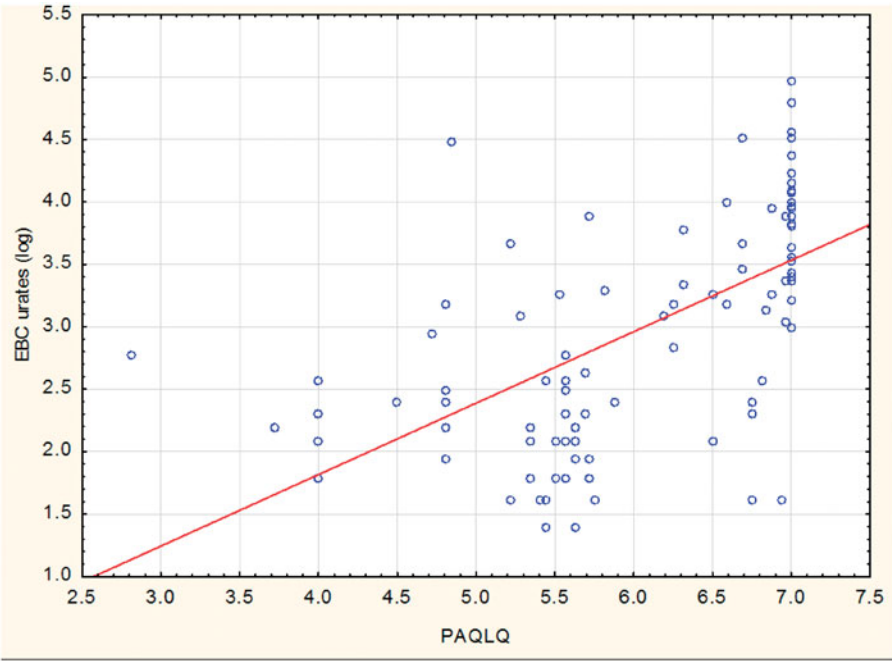


Figure 9. Relationship between exhaled breath condensate (EBC) urates and time from last exacerbation (TLE; $r = 0.572$, $p < 0.0001$); categories 0–5 represent uncontrolled asthma (0 – uncontrolled with no exacerbations, 1 – last exacerbation during preceding month; 2 – last exacerbation 1–3 months ago; 3 – last exacerbation >3 to 6 months ago; 4 – last exacerbation >6 to 12 months ago, 5 – last exacerbation >12 months ago; category 6 represents controlled asthma).

In our work, EBT was increased in uncontrolled asthmatics and significantly associated with hs-CRP, *prn* salbutamol use, ACT and PAQLQ. In addition to local inflammation, in asthma, a low grade systemic inflammation that can be assessed using serum hs-CRP is also present [9,30]. Hs-CRP was increased in uncontrolled asthmatics and associated with asthma severity, *prn* salbutamol use and acute exacerbations [36,37]. In our study, serum hs-CRP was also (insignificantly) increased in uncontrolled asthma and inversely correlated with FEV₁ and a post-bronchodilator FEV₁/FVC. Low

post-bronchodilator FEV₁/FVC was proposed as a marker of airway remodeling [38] and an inverse correlation between hs-CRP and a post-bronchodilator FEV₁/FVC could suggest a relationship between systemic inflammation and airway remodeling. In our study, being the first observation of that kind, we found an association between hs-CRP and EBT as a biomarker of local inflammation in asthmatics. We can hypothesize that systemic inflammation in asthmatics is associated not only with the current level of local chronic inflammation (assessed by EBT and FEV₁) but also with the long lasting pulmonary damage assessed by post-bronchodilator FEV₁/FVC.

In our study, a combination of biomarkers for the evaluation of underlying airway inflammation and asthma control showed satisfying diagnostic performance that was slightly but not significantly better than EBC urates alone and a significantly larger study would be needed to evaluate the possible additional benefit. However, biomarkers used are not disease specific and can reflect other diseases and conditions except asthma so serial measurements of biomarkers maybe could be more informative about the diagnostic value of these biomarkers in the assessment of asthma control. Hence, we can conclude that these non-invasive, simple and low-cost biomarkers that could be assessed during the outpatient visit certainly could be useful as additional parameters of underlying airway inflammation and asthma control.

We are aware of certain limitations of this study like the lack of data on other systemic and local inflammatory and oxidative stress biomarkers. Furthermore, this was not a longitudinal study with repeated measurements of biomarkers with the consecutive monitoring of asthma control. In addition, there are several factors that could confound study results like wide age range, gender difference, BMI, difference in atopic status, different physical activity, passive smoking and nutrition, although for most of them the groups were well matched. Then, the assessment of associations between biomarkers and measures of asthma control was not a

primary goal of study and were done observationally with no correction for multiple testing. We have to be very careful in interpretation of EBC urates' measures due to the fact that EBC is very diluted fluid of airway secretion, i.e. more than 1000-fold dilution [39]. It is important to note that approximately 60% of an anti-oxidative potential of RELF comes from urates. As there are deficient data on EBC urates concentrations in the available literature, interpretation of our results is very difficult and can be based on the conclusion emerging from BALF studies. The discrepancy is noticed between our EBC urates concentrations and calculated urates concentrations based on BALF studies [40–42], although EBC urates concentrations in our subjects were comparable with EBC urates concentrations measured in a study of another investigators group in our hospital [11]. As normal uric acid concentrations in BALF were estimated to be about 1 $\mu\text{mol/L}$ [40] uric acid concentration in airway secretion in healthy children can be estimated at $\sim 100 \mu\text{mol/L}$. Hence, it would be expected that EBC concentrations to be on the order of 0.1 $\mu\text{mol/L}$ since airway secretions in EBC are diluted 1000-fold or more. However, our EBC urates concentrations ranging between 10 and 45 $\mu\text{mol/L}$ were 100+ times higher than expected. This discrepancy is hard to explain but regarding the fact that the EBC dilution markers, dilution itself and the source of EBC are still not known, urates represented in EBC may differ from those in BALF. The source of airway secretions in EBC is still a matter of dispute but has been postulated to represent entraining of airway surface liquid microdroplets generated from turbulent airway flow [39]. Also, increased metabolism of purines in EBC occurring during specimen processing, could also account for the differences [28]. In addition, the variable dilution of the resident lung epithelial lining fluid poses the fundamental problems for the standardized expression and interpretation of EBC and BALF findings [39,43]. Furthermore, since we did not directly assess an established biomarker of oxidative stress, we cannot rule out the possibility that the decreased urates reflect some other aspect of asthma. Hence, more sensitive method as well as confident dilution marker is needed for more precise determination of uric acid in EBC.

Study results have demonstrated that non-invasive local biomarkers of oxidative stress, endogenous acidification and inflammation, especially EBC urates, might become clinically useful markers of underlying airway inflammation and asthma control reflecting a multifactorial pathophysiology of asthma. Significant correlations of serum hs-CRP with EBT, and of EBC urates with EBC pH may reflect an association of inflammation, oxidative and acid stress in asthmatics. To further evaluate the clinical utility of local and systemic biomarkers in monitoring the control in childhood asthma, more studies with repeated measurements of biomarkers together with the concurrent monitoring of asthma control are needed.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. The results presented have been obtained in the scope of a scientific project No. 277-2770968-0963, entitled Early Indicators of Development of Allergic Diseases in Children, carried out with support from the Ministry of Science, Education and Sports of the Republic of Croatia.

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