

## ORIGINAL ARTICLE: ASTHMA

# Urinary and exhaled biomarkers of exercise-induced bronchoconstriction in atopic asthmatic children

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## Abstract

**Background:** Exercise-induced bronchoconstriction (EIB) reflects poor asthma control. Assessing noninvasive biomarkers associated with EIB could help to monitor patients in the pediatric age.

**Aims:** To test exhaled and urinary biomarkers for assessing EIB in atopic asthmatic children.

**Methods:** In 45 atopic patients ( $11.1 \pm 1.8$  years, 25 males) we measured the fractional exhaled nitric oxide ( $FE_{NO}$ ), its alveolar ( $CaNO$ ), and bronchial ( $J'awNO$ ) components corrected for the trumpet shape of the airways and axial NO diffusion (TMAD), concentrations of urinary adenosine and 8-hydroxy-2'-deoxyguanosine (8-OxodG), blood eosinophils count, total immunoglobulin E, skin prick tests, and baseline spirometry before a treadmill exercise challenge. Forty healthy control subjects participated solely to baseline measurements.

**Results:** Patients yielded higher  $FE_{NO}$  and urinary adenosine concentrations than healthy controls. After the challenge, 18 patients (40%) had EIB; these patients had higher levels of  $CaNO$ ,  $CaNO$  TMAD, and urinary adenosine than patients without EIB. Baseline spirometry,  $FE_{NO}$ ,  $JawNO$ ,  $JawNO$  TMAD, urinary 8-OxodG, allergy, and blood eosinophil counts were found similar in both groups. In multiple linear regression, the fall in  $FEV_1$  was explained by  $CaNO$  TMAD, urinary adenosine and blood eosinophil count, whereas the fall in  $FEF_{25-75}$  was explained by  $CaNO$  TMAD and blood eosinophil count. Both  $CaNO$  TMAD  $\geq 10.5$  ppb and urinary adenosine  $\geq 406$  nmol/mmol Cr predicted a fall in  $FEV_1 \geq 10\%$ , while only  $CaNO$  TMAD  $\geq 10.5$  ppb predicted a fall in  $FEF_{25-75} \geq 26\%$ .

**Conclusion:** Concentrations of peripheral airway NO are complementary with urinary adenosine for assessing EIB and promising tools of asthma control in pediatric patients with the atopic phenotype.

## KEYWORDS

adenosine, allergy, asthma, eosinophils, guanosine, nitric oxide, pediatrics, treadmill test, urine

## 1 | INTRODUCTION

Asthma is associated with low physical activity in children; exercise intolerance becomes more evident with loss of disease

control.<sup>1-3</sup> The bronchial challenge with exercise is indeed a good way to assess asthma control<sup>2</sup>; however, exercise testing is not practical to be repeated in the routine. Alternatively, noninvasive and easy-to-perform biomarkers may be used as

surrogates of exercise-induced bronchoconstriction (EIB) in pediatric patients.<sup>3</sup>

Research of biomarkers involved in mechanisms of EIB is challenging, particularly because asthma is a heterogeneous disease. The fractional concentration of exhaled nitric oxide (FE<sub>NO</sub>), a biomarker of eosinophilic airway inflammation, is related with the airway response to exercise in atopic-asthmatic children.<sup>3</sup> Increased proximal (bronchial) NO production or increases in both bronchial and peripheral (bronchiolar-alveolar) NO has been reported in these patients.<sup>4,5</sup> High concentrations of cysteinyl-leukotrienes and 8-isoprostane in the exhaled breath condensate (EBC), biomarkers of airway inflammation and oxidant stress respectively, have also been found predictive of EIB.<sup>6,7</sup>

In addition to analyses of respiratory samples in the gas-phase or EBC, there is an increasing interest into quantify inflammatory-related metabolites in urine samples of asthmatic patients.<sup>8</sup> The usefulness of measuring these urinary metabolites as biomarkers of EIB, remains to be investigated.

Recent data suggest that some derivatives of the two nucleobases, adenine, and guanine, have a key role in the inflammatory cascade. Examples of these derivatives are adenosine and 8-hydroxy-2'-deoxyguanosine (8-OxodG). Extracellular adenosine derives from inflammation-induced breakdown of adenosine triphosphate (ATP); in these conditions, adenosine up regulates the Th2 response but also induces immunosuppression and tissue repair, when healthy cells become collaterally damaged.<sup>9</sup> The guanosine-oxidized product 8-OxodG is released from the nucleic acid DNA by the action of several reactive oxygen species originated during inflammatory reactions but also in other endogenous or exogenous sources.<sup>10</sup> Most studies on oxidized nucleosides have been focused in cancer, aging, and diabetes.<sup>10,11</sup>

Urinary concentrations of adenosine have been measured in healthy children,<sup>12,13</sup> and few patients with miscellaneous conditions such as inherited metabolic disorders, cardiomyopathy, and respiratory failure.<sup>13</sup> Urinary measurements of 8-OxodG have been conducted in healthy children,<sup>14</sup> contemporary patients with cystic fibrosis,<sup>15</sup> and subjects exposed to environmental pollutants,<sup>16,17</sup> including asthmatic patients exposed to tobacco smoke.<sup>17</sup>

Whereas some knowledge of FE<sub>NO</sub> and partitioned-NO is available in relation with EIB, no information exists on the role of urinary purine metabolites in assessing EIB in asthmatic children.

We aimed to test exhaled and urinary biomarkers for assessing the airway response to exercise in a common phenotype of pediatric asthma. To do so, we measured partitioned exhaled NO and urinary nucleosides together with lung function, skin prick tests, and blood eosinophils in atopic asthmatic children.

## 2 | MATERIALS AND METHODS

### 2.1 | Subjects

Forty-five atopic outpatients with mild intermittent asthma attending the pulmonary section of our Pediatric Unit (Sant'Andrea Hospital,

Rome, Italy) were enrolled to perform a bronchial challenge with exercise. They were classified following the Global Initiative for Asthma guidelines.<sup>18</sup> All patients had a previously documented bronchial reversibility to salbutamol ( $\geq 12\%$ ) and/or a fall in FEV<sub>1</sub> of at least 12% after exercise testing. Inclusion criteria were a pre-exercise FEV<sub>1</sub>  $\geq 80\%$  of the predicted value the day of exercise testing, absence of respiratory infections in the previous 4 weeks and avoidance of asthma therapy (oral or inhaled corticosteroids or Montelukast) in the last 2 weeks. The consensus-based GINA symptom control tool was used to involve patients with "well controlled" or "partly controlled" symptoms as well as to exclude those "uncontrolled" at recruitment.<sup>18</sup>

Forty healthy control subjects were recruited from two schools placed in the north of Rome (C. Pavese and I. Alpi); eligible subjects were invited to participate. Under the approval, school children underwent a medical visit, skin prick tests for common allergens, FE<sub>NO</sub> measurements, followed by a baseline spirometry. In the same session, they collected urine samples and their parents completed a questionnaire on respiratory health as previously described.<sup>19</sup> Subjects were included as healthy controls if they had a negative history for atopic disease or chronic respiratory symptoms, a negative skin prick test and a normal spirometry. Healthy children did not perform partitioned-NO collection, blood samples nor exercise testing.

Parents were asked to sign a written informed consent; the study was approved by the Ethical Review Board of our Institution.

### 2.2 | Study design

All measurements were obtained in the morning during a single session. Patients were assessed for current respiratory symptoms and asthma medications underwent a medical visit and collected urine samples. Blood samples, skin prick testing and FE<sub>NO</sub> measurements were taken before they did a baseline spirometry and a treadmill exercise testing. Parents of the atopic-asthmatic children were asked to provide data on smoking habits and child's practice of sports outside school. Physician's diagnosis of allergic rhinitis and asthma episodes in the past 12 months were also recorded. Reported exercise-induced cough, dyspnea, and chest pain/tightness were asked to the child and recorded affirmative if perceived with discomfort.

### 2.3 | Urine samples

One spot urine sample was collected for each subject in a sterile polypropylene container, transported to the laboratory and aliquoted in several 2 mL probes. Samples were frozen at  $-80^{\circ}\text{C}$  within 2 hours of collection, until analyses. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was used to analyze the concentration of adenosine and 8-OxodG in urine samples. Urinary creatinine concentrations were measured with the Jaffé method using an appropriate reagent (DiaSys diagnostic system GmbH, Alte Strasse 9 65558 Holzheim, Germany). The measured creatinine

concentration was used to normalize concentrations of adenosine and 8-OxodG<sup>10-12</sup>; both urinary metabolites were expressed as nanomoles metabolite/millimoles creatinine (nmol/mmol).

## 2.4 | Detection and quantification of adenosine and 8-OxodG in LC-MS/MS

Urine samples were processed using a deproteinizing solution containing the internal standard. The clean upper layers were injected into the chromatographic system. The high-performance liquid chromatography analysis was performed using an Agilent liquid chromatography system series 1100 (Agilent Technologies). Chromatographic separation was performed using a reversed phase column. The mass spectrometry method was performed on a 3200 triple quadrupole system (Applied Biosystems, Foster City, CA) equipped with a Turbo ion spray source. The detector was set in the positive ion mode. The instrument was set in the multiple reaction monitoring mode. The MS/MS ion transitions monitored were 136.1 and 119.1 for adenosine, 168.1 and 140.1 for 8-OxodG. Data were acquired and processed with Analyst 1.5.1 software.

## 2.5 | Blood measurements

Blood samples were obtained to measure the eosinophil blood count and serum total IgE concentrations. An automatic blood cells counter Sysmex XT2100 (Sysmex America, Mundelein, IL) was used to measure white cells; serum IgE was assessed by ImmunoCAP (Thermo Fisher Scientific, Phadia, Uppsala, Sweden).

## 2.6 | Skin prick tests

Skin prick tests for common inhaled and food allergens were performed (ALK-Abellö, Milan, Italy) including house dust mites (Dpt, and Dpf), cat and dog fur, *Alternaria alternata*, mixed grass pollens and mixed tree pollens, *Parietaria judaica*, egg (white and yolk), and milk. Histamine 0.1 mg/mL and glycerol solution were used as positive and negative controls, respectively. A wheal  $\geq 3$  mm after subtraction of the negative control was considered as positive.<sup>20</sup> The number of positive skin reactions was recorded.

## 2.7 | FE<sub>NO</sub> and partitioned-NO measurements

An electrochemical apparatus (HyPair FE<sub>NO</sub>, Medisoftware Group, Sorinnes, Belgium) was used to measure FE<sub>NO</sub>. Children were asked to perform single-breath maneuvers at a constant expiratory pressure (10 cm H<sub>2</sub>O) and flow (50 mL/s).<sup>21</sup> Expiratory maneuvers were repeated until at least two measurements agreed within 10% and their mean value was calculated as recommended.<sup>21</sup>

To estimate the peripheral and proximal airway contributions of exhaled NO, we used the two-compartment model as described by Tsoukias and George.<sup>22</sup> Subjects did duplicate exhalation maneuvers at three different target flows (50 mL/s, 100 mL/s, and 150 mL/s). The product of each exhaled flow by the NO concentration in ppb (ie, the

NO output, nL/s) was then calculated and plotted in a linear regression where the intercept (J'awNO, nL/s) is the maximum NO flux from airways and the slope (CaNO, ppb) is the small airway and alveolar NO concentration.<sup>22</sup> These two flow-independent NO parameters were corrected for the trumpet shape of the airway tree and the gas-phase airway diffusion of NO, ie, the Trumpet Model of airway diffusion, TMAD.<sup>23</sup> The applied corrections for the range of target steady-state flows we used were as follows,

$$J'awNO_{TMAD} = J'awNO \times 1.9$$

$$CaNO_{TMAD} = CaNO - J'awNO / 0.53$$

Where J'awNO TMAD and J'awNO are expressed in nL/s, CaNO and CaNO TMAD are ppb, and 0.53 are L/s.

## 2.8 | Spirometry

Spirometric measurements were recorded using a Biomedin Spirometer (Biomedin Srl, Padua, Italy). At least three acceptable maneuvers of forced vital capacity (FVC) were required to select two repeatable flow volume curves<sup>24</sup>; dynamic volumes and flows were expressed as a percentage of the predicted values.<sup>25</sup>

## 2.9 | Exercise testing

After a baseline spirometry, patients underwent an incremental treadmill exercise test as described elsewhere.<sup>7</sup> They ran at 6 km/h with a pendant 10% until reaching the target heart rate over 85% of predicted maximum, calculated as 220- age in years.<sup>26</sup> Stable room temperature (20-24°C) and relative humidity (50% and 60%) were allowed during the procedure. Following exercise, spirometry was repeated at 1, 5, 10, 15 and 20 minutes and the lowest FEV<sub>1</sub> value was used to calculate the percentage of FEV<sub>1</sub> fall from baseline. EIB was defined as a postexercise fall in FEV<sub>1</sub> of at least 10%<sup>26</sup>; peripheral airway narrowing was defined as a postexercise fall in FEF<sub>25-75</sub> of at least 26%.<sup>27</sup>

## 2.10 | Statistical analysis

Continuous variables were assessed for normal distribution using a Kolmogorov-Smirnov test; normally distributed variables were expressed as means and SD whereas those skewed were expressed as geometric means and 95% confidence intervals (95% CI). To compare urinary biomarkers by occurrence of EIB, the sample size was calculated for a difference of means in normalized adenosine concentrations (nmol adenosine/mmol creatinine) of 0.10 nmol/mmol, an SD of 0.12 nmol/mmol, with a power (1- $\beta$ ) of 80% and a level of significance of 5% as described elsewhere<sup>7</sup>; these calculations yielded two groups (patients with EIB and patients without EIB) with at least 18 subjects each.

Receiver operating characteristic (ROC) curves were used to describe the relationship between sensitivity and 1-specificity for measurements predictive of EIB; areas under curves (AUCs) were calculated.<sup>20</sup> The Mann-Whitney U test was used to assess differences for continuous variables between two groups, whereas

contingency tables ( $\chi^2$ ) with Fisher's correction was used to compare frequencies. The Pearson's correlation coefficient or the non-parametric Spearman's Rho were used to correlate continuous variables, as needed. Multiple linear regressions (stepwise method) were performed with the postexercise fall of either FEV<sub>1</sub> or FEF<sub>25-75</sub> as dependent variables against several potentially influencing (independent) variables, selected on the basis of either a significant correlation with the dependent variables or significant differences between categories (eg the fall of FEV<sub>1</sub> between groups without vs with reported cough with exercise)<sup>19</sup>. A statistical software (SPSS version 24, Chicago, IL) was used for calculations. Statistical significance was accepted for " $P$ " < .05.

### 3 | RESULTS

Forty-five atopic-asthmatic patients completed all measurements; their FE<sub>NO</sub> and urinary adenosine concentrations were found increased as compared with those of the 40 healthy controls. In contrast, lung function, urinary 8-OxodG concentrations, parental smoking habits, sports practice, and antropometric characteristics did not discriminate patients from healthy subjects (Table 1).

The exercise challenge induced EIB in 18/45 (40%) of the asthmatic patients. These 18 patients more frequently reported a history of exercise-induced cough (but not dyspnea or chest pain) than their 27 counterparts whose exercise challenge yielded negative. Cough with exercise was reported in 16/18 patients with EIB (sensitivity 88.9%); instead, negative history of cough was found in only 11/27 (specificity 40.7%) of the patients without EIB.

High levels of CaNO, CaNO TMAD, and urinary adenosine distinguished patients with EIB from those without EIB (Table 2). Also total serum IgE levels of subjects with EIB were found elevated but not significantly higher than those of subjects without EIB ( $P$  = .067 by Mann-Whitney test; Table 2). In contrast, baseline lung function, FE<sub>NO</sub>, JawNO, JawNO TMAD, urinary 8-OxodG concentrations, and blood eosinophil counts were found similar in both groups of exercise challenge outcomes. The proportion of subjects with "partially controlled" vs "well controlled" asthma symptoms (GINA symptom control tool), was higher in the group with EIB than in the group without, though this comparison did not achieve statistical significance (EIB, 10/18: 55.5% vs without EIB, 10/27: 37.0%;  $P$  = .241).

In the whole group of 45 asthmatic patients, CaNO, CaNO TMAD, and blood eosinophil counts correlated with the postexercise fall of both FEV<sub>1</sub> and FEF<sub>25-75</sub>. Urinary adenosine concentrations correlated only with the fall in FEV<sub>1</sub>, whereas total serum IgE levels correlated only with the fall in FEF<sub>25-75</sub> (Table 3; Figure 1).

A multiple-stepwise linear regression with the postexercise fall in either FEV<sub>1</sub> or FEF<sub>25-75</sub> as dependent against potentially explanatory measurements and anthropometric characteristics yielded two different predictive models in our asthmatic patients (FEV<sub>1</sub> fall:  $r$  = 0.66,  $P$  = .000; FEF<sub>25-75</sub> fall:  $r$  = 0.65,  $P$  = .000) as follows:

**TABLE 1** Subjects characteristics

| Variables                        | Healthy (n = 40) | Asthmatic (n = 45) |
|----------------------------------|------------------|--------------------|
| Age, yr                          | 11.0 ± 1.0       | 11.1 ± 1.8         |
| Sex, M/F                         | 20/20            | 25/20              |
| Weight, kg                       | 40.2 ± 6.9       | 44.0 ± 12.5        |
| Height, cm                       | 145.2 ± 9.0      | 147.9 ± 13.7       |
| BMI percentile                   | 68.4 ± 20.8      | 67.8 ± 27.6        |
| Passive smoke at home, n (%)     | 13 (32.5)        | 20 (44.4)          |
| Sports outside school, n (%)     | 30 (75.0)        | 30 (66.7)          |
| Spirometry and exhaled NO levels |                  |                    |
| FEV <sub>1%</sub>                | 98.5 ± 10.2      | 98.0 ± 10.8        |
| FVC %                            | 98.5 ± 18.0      | 98.8 ± 10.5        |
| FEV <sub>1</sub> /FVC %          | 89.8 ± 5.8       | 88.6 ± 6.9         |
| FEF <sub>25-75%</sub>            | 92.4 ± 19.3      | 87.9 ± 22.4        |
| FE <sub>NO</sub> , ppb           | 16.1 (14.4–18.0) | 34.7 (29.5–40.8)** |
| Urinary metabolites              |                  |                    |
| 8-OxodG/Cr, nmol/mmol            | 1.84 ± 0.96      | 1.60 ± 0.76        |
| Adenosine/Cr, nmol/mmol          | 258.2 ± 106.1    | 351.7 ± 128.5**    |

Note: Continuous variables are expressed as arithmetic means ± SD; fractional concentrations of exhaled nitric oxide (FE<sub>NO</sub>) are expressed as geometric means (95% confidence interval). Urinary concentrations of 8-Hydroxy-2'-deoxyguanosine (8-OxodG) and adenosine are normalized by urinary creatinine.

Abbreviations: NO, nitric oxide; 8-OxodG, 8-hydroxy-2'-deoxyguanosine. \*\* $P$  < .005 by Mann-Whitney test.

FEV<sub>1</sub> fall = 14.378 + (CaNO TMAD × −0.960) + (blood eosinophil count × −0.013) + (urinary adenosine/creatinine × −0.036).

FEF<sub>25-75</sub> fall = −0.873 + (blood eosinophil count × −0.022) + (CaNO TMAD × −1.300).

Where CaNO TMAD is expressed in ppb, blood eosinophil count as cells/mm<sup>3</sup> and urinary adenosine as normalized urinary adenosine by urinary creatinine, nmol/mmol.

Values above the percentile 70 of both CaNO TMAD (10.5 ppb) and urinary adenosine (406 nmol/mmol Cr) predicted a fall in FEV<sub>1</sub> ≥10%, especially those of adenosine (sensitivity 61.1%, specificity 92.6%, positive predictive value 84.6%, negative predictive value 78.1%). Conversely, CaNO TMAD levels above the 70 percentile better predicted a fall in FEF<sub>25-75</sub> ≥26% (Table 4; Figure 2).

### 4 | DISCUSSION

Concentrations of the peripheral airway NO (CaNO, and CaNO TMAD), and urinary adenosine were useful for assessing the airway response to exercise in our asthmatic patients with the atopic phenotype. Both CaNO TMAD and urinary adenosine predicted the fall in FEV<sub>1</sub>, while CaNO TMAD predicted the fall in FEF<sub>25-75</sub>. The increased blood eosinophil count, a characteristic biomarker of atopic inflammation, was a constant predictor of bronchial hyperresponsiveness whichever the airway-functional level (fall in FEV<sub>1</sub> or FEF<sub>25-75</sub>) of

**TABLE 2** Comparison between asthmatic patients without and with exercise-induced bronchoconstriction (EIB)

| Variables                                        | Without EIB<br>(n = 27) | EIB (n = 18)           |
|--------------------------------------------------|-------------------------|------------------------|
| Age, yr                                          | 11.1 ± 1.8              | 11.1 ± 1.9             |
| Sex, M/F                                         | 15/12                   | 10/8                   |
| Weight, kg                                       | 43.8 ± 12.5             | 44.3 ± 12.7            |
| Height, cm                                       | 148.1 ± 14.1            | 147.7 ± 13.5           |
| BMI percentile                                   | 67.8 ± 27.9             | 67.8 ± 28.0            |
| Passive smoke at home,<br>n (%)                  | 11 (40.7)               | 9 (50.0)               |
| Sports outside school,<br>n (%)                  | 17 (63.0)               | 13 (72.2)              |
| Symptoms last 12 mo                              |                         |                        |
| Rhinitis, n (%)                                  | 14 (52.0)               | 10 (55.6)              |
| ≥1 asthma episode, n (%)                         | 17 (63.0)               | 15 (83.3)              |
| Asthma therapy >2 mo/yr                          |                         |                        |
| Inhaled corticosteroids                          | 4 (14.8)                | 1 (5.6)                |
| Montelukast                                      | 3 (11.1)                | 4 (22.2)               |
| Reported symptoms with<br>exercise               |                         |                        |
| Cough, n (%)                                     | 16 (59.3)               | 16 (88.9)*             |
| Dyspnea, n (%)                                   | 23 (85.2)               | 17 (94.4)              |
| Chest pain, n (%)                                | 10 (37.0)               | 8 (44.4)               |
| Serum IgE levels, UI/dL                          | 176.2<br>(95.3–325.9)   | 329.4<br>(154.5–702.3) |
| Allergen skin prick wheals<br>≥3mm, n            | 3.2 ± 1.4               | 3.2 ± 1.7              |
| Blood eosinophil count,<br>cells/mm <sup>3</sup> | 333.1<br>(243.6–455.4)  | 452.5<br>(295.1–694.0) |
| Baseline spirometry                              |                         |                        |
| FEV <sub>1</sub> %                               | 97.9 ± 11.0             | 98.1 ± 10.7            |
| FVC %                                            | 96.7 ± 10.1             | 101.8 ± 10.6           |
| FEV <sub>1</sub> /FVC %                          | 90.2 ± 6.3              | 86.7 ± 7.1             |
| FEF <sub>25%-75%</sub>                           | 92.3 ± 24.7             | 81.4 ± 17.1            |
| Exhaled and partitioned-NO<br>levels             |                         |                        |
| FE <sub>NO</sub> , ppb                           | 31.9 (25.7–39.5)        | 39.3 (30.3–51.2)       |
| JawNO, nL/s                                      | 1.19 (0.94–1.51)        | 1.19 (0.80–1.76)       |
| JawNO TMAD, nL/s                                 | 2.26 (1.78–2.87)        | 2.26 (1.52–3.34)       |
| CaNO, ppb                                        | 8.7 (7.1–10.8)          | 12.9 (9.6–17.2)*       |
| CaNO TMAD, ppb                                   | 5.8 (4.4–7.8)           | 9.3 (6.1–14.3)*        |
| Urinary metabolites                              |                         |                        |
| 8-OxodG/Cr, nmol/mmol                            | 1.45 ± 0.74             | 1.70 ± 0.80            |
| Adenosine/Cr, nmol/mmol                          | 313.7 ± 103.2           | 408.6 ± 143.9*         |

Note: Continuous variables are expressed as arithmetic means ± SD, or as geometric means (95% CI).

"Rhinitis" and "Asthma episodes" are physician's diagnosis of allergic rhinitis and asthma exacerbations in the last 12 mo.

Abbreviations: BMI, basal metabolic rate; CaNO: alveolar NO; FE<sub>NO</sub>, fractional exhaled nitric oxide; JawNO, maximal NO production from

airway diffusion; JawNO TMAD and CaNO TMAD, corrected JawNO and CaNO for the trumpet model of airway diffusion;<sup>23</sup> NO, nitric oxide; TMAD, trumpet model of airway diffusion.

Urinary concentrations of 8-Hydroxy-2'-deoxyguanosine (8-OxodG) and adenosine are normalized by urinary creatinine.

\*P < .05 by Fisher's corrected  $\chi^2$  (cough) or by Mann-Whitney test (CaNO and adenosine/Cr).

**TABLE 3** Spearman's Rho correlations with the postexercise fall in spirometric variables in the 45 asthmatic-atopic children

| Variables                                        | Percent fall in<br>FEV <sub>1</sub> |       | Percent fall in<br>FEF <sub>25-75</sub> |       |
|--------------------------------------------------|-------------------------------------|-------|-----------------------------------------|-------|
|                                                  | r                                   | P     | r                                       | P     |
| Age, yr                                          | −0.03                               | 0.860 | 0.22                                    | 0.141 |
| Weight, kg                                       | −0.16                               | 0.282 | 0.07                                    | 0.638 |
| Height, cm                                       | 0.07                                | 0.658 | 0.14                                    | 0.355 |
| BMI percentile                                   | −0.08                               | 0.581 | −0.08                                   | 0.588 |
| Serum IgE levels, UI/dL                          | −0.29                               | 0.073 | −0.44                                   | 0.005 |
| Allergen skin prick<br>wheals ≥3 mm, n           | 0.07                                | 0.647 | 0.01                                    | 0.969 |
| Blood eosinophil count,<br>cells/mm <sup>3</sup> | −0.35                               | 0.024 | −0.46                                   | 0.002 |
| FEV <sub>1</sub> %                               | −0.00                               | 0.978 | −0.00                                   | 0.994 |
| FVC %                                            | −0.06                               | 0.680 | −0.01                                   | 0.936 |
| FEF <sub>25-75</sub> %                           | 0.08                                | 0.586 | 0.21                                    | 0.162 |
| FE <sub>NO</sub> , ppb                           | −0.25                               | 0.094 | −0.28                                   | 0.060 |
| JawNO, nL/s                                      | −0.12                               | 0.448 | −0.14                                   | 0.370 |
| JawNO TMAD, nL/s                                 | −0.12                               | 0.448 | −0.14                                   | 0.370 |
| CaNO, ppb                                        | −0.49                               | 0.001 | −0.47                                   | 0.001 |
| CaNO TMAD, ppb                                   | −0.49                               | 0.001 | −0.47                                   | 0.001 |
| Urinary 8-OxodG/Cr,<br>nmol/mmol                 | −0.27                               | 0.077 | 0.01                                    | 0.927 |
| Urinary adenosine/Cr,<br>nmol/mmol               | −0.43                               | 0.003 | −0.20                                   | 0.189 |

Note: Urinary concentrations of 8-Hydroxy-2'-deoxyguanosine (8-OxodG) and adenosine are normalized by urinary creatinine.

Abbreviations: BMI, basal metabolic rate; CaNO, alveolar NO; FE<sub>NO</sub>, fractional exhaled nitric oxide; JawNO, maximal NO production from airway diffusion; JawNO TMAD and CaNO TMAD, corrected JawNO and CaNO for the trumpet model of airway diffusion;<sup>23</sup> TMAD, trumpet model of airway diffusion.

these children was considered. To this regard, our findings confirm the relationship between blood eosinophil counts and EIB.<sup>28</sup>

Several reports have suggested that increased FE<sub>NO</sub> could be a good surrogate of EIB, based on the correlation between baseline FE<sub>NO</sub> and the degree of the airway response to exercise.<sup>29–31</sup> However, asthmatic patients with EIB had significantly higher baseline FE<sub>NO</sub> than healthy control subjects but not than asthmatic patients without EIB in two of these reports.<sup>30,31</sup> Neither we found different FE<sub>NO</sub> between patients with vs without EIB in a previous

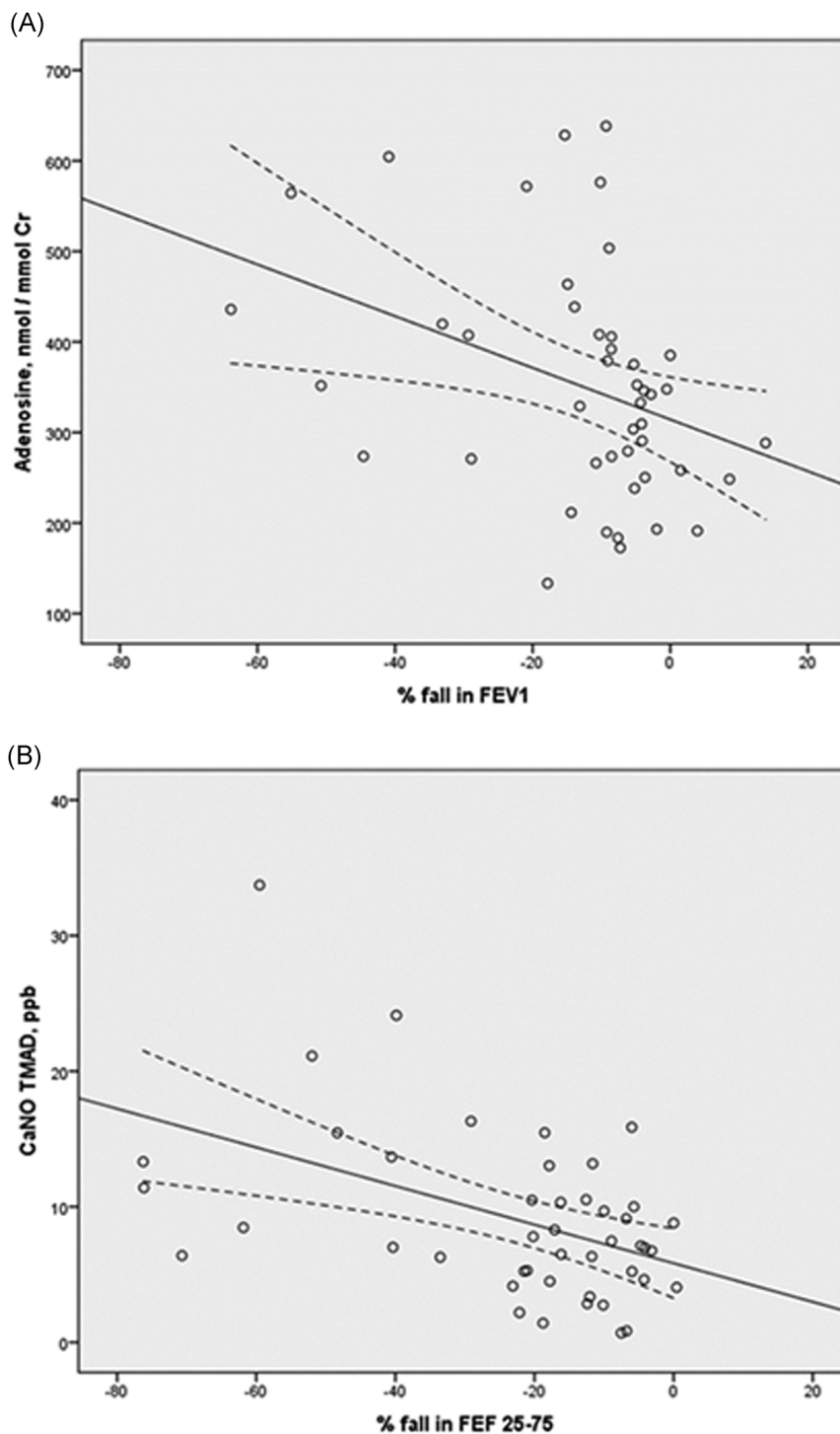


**FIGURE 1** Correlations between baseline biomarkers and the postexercise decline in spirometric parameters. Lines are the estimated slope and 95% confidence interval (CI). A, Normalized concentrations of urinary adenosine by urinary creatinine (nanomoles/millimoles) vs the percent fall in FEV<sub>1</sub>,  $r = -0.43$ ,  $P = .003$ ; slope:  $Y = 314.3 - 2.85x$  (95% CI: -5.11 to -0.60). B, Alveolar nitric oxide concentrations corrected for the Trumpet Model of Airway Diffusion (CaNO TMAD, ppb) vs the percent fall in FEF<sub>25-75</sub>,  $r = -0.47$ ,  $P = .001$ ; slope:  $Y = 5.83 - 0.14x$  (95% CI: -0.23 to -0.06)

study.<sup>7</sup> Because most of the differences between studies relies in the proportion of atopic subjects and the current corticosteroid therapy, in the present study we selected solely atopic patients whose controller therapy could be avoided in the last 2 weeks. Nonetheless, we could not find FE<sub>NO</sub> as a useful tool for assessing EIB.

Baseline FE<sub>NO</sub> has been reported as better correlated with the postexercise fall in FEF<sub>25-75</sub> than with the corresponding fall in FEV<sub>1</sub><sup>32</sup>; these correlations were similar, though statistically borderline, in our study. Instead, baseline CaNO and CaNO TMAD closely

correlated with the postexercise fall of both spirometric parameters, as we discussed below.

Two pediatric studies have assessed partitioned-airway NO exchange parameters before an exercise challenge test.<sup>4,5</sup> Linkosalo et al<sup>4</sup> estimated both uncorrected and corrected parameters for backward axial NO diffusion in subjects with asthma-like symptoms; JawNO, JawNO TMAD, and CaNO (but not CaNO TMAD) were higher in subjects who develop EIB as compared with those without. The authors suggested that CaNO could be falsely increased in

**TABLE 4** Prediction of EIB by levels of urinary adenosine and alveolar nitric oxide (CaNO TMAD) using two different spirometric criteria for a positive exercise challenge

|                                                      | Percentiles (concentrations)     |          |          |                 |           |           |
|------------------------------------------------------|----------------------------------|----------|----------|-----------------|-----------|-----------|
|                                                      | Urinary adenosine (nmol/mmol Cr) |          |          | CaNO TMAD (ppb) |           |           |
|                                                      | 60 (366)                         | 70 (406) | 80 (438) | 60 (9.0)        | 70 (10.5) | 80 (13.3) |
| EIB: post-exercise fall in FEV <sub>1</sub> ≥ 10%    |                                  |          |          |                 |           |           |
| Sensitivity                                          | 61.1                             | 61.1     | 38.9     | 50.0            | 50.0      | 38.9      |
| Specificity                                          | 74.1                             | 92.6     | 92.6     | 66.7            | 85.2      | 92.6      |
| PPV                                                  | 61.1                             | 84.6     | 77.8     | 50.0            | 69.2      | 77.8      |
| NPV                                                  | 74.1                             | 78.1     | 69.4     | 66.7            | 71.9      | 69.4      |
| EIB: postexercise fall in FEF <sub>25-75</sub> ≥ 26% |                                  |          |          |                 |           |           |
| Sensitivity                                          | 58.3                             | 58.3     | 33.3     | 66.7            | 66.7      | 58.3      |
| Specificity                                          | 66.7                             | 81.8     | 84.8     | 69.7            | 84.8      | 93.9      |
| PPV                                                  | 38.9                             | 53.8     | 44.4     | 44.4            | 61.5      | 77.8      |
| NPV                                                  | 81.5                             | 84.3     | 77.8     | 85.2            | 87.5      | 86.1      |

Note: Urinary concentrations of adenosine are normalized by urinary creatinine. CaNO TMAD (ppb): alveolar nitric oxide corrected for the trumpet model of airway diffusion.<sup>23</sup>

Abbreviations: EIB, exercise-induced bronchoconstriction; NPV, negative predictive value; PPV, positive predictive value.

patients with high NO flux; thereby they attributed a better value to JawNO to predict EIB.<sup>4</sup> Chinelato et al<sup>5</sup> measured uncorrected CaNO and JawNO in allergic children with intermittent asthma; these parameters correlated with the severity of EIB suggesting the presence of inflammation in both central and peripheral airways. In contrast, we found that only CaNO TMAD predicted the postexercise decay of both FEV<sub>1</sub> and FEF<sub>25-75</sub>, suggesting that peripheral airway inflammation occurs in atopic patients prone to EIB. Differences in the flow range used, 50 to 300 mL/s<sup>4</sup> and 10 to 200 mL/s,<sup>5</sup> prevent us to compare our results with those of the preceding studies.

Two factors are capable to affect peripheral NO measurements: the exhaled flow rate and the patient's degree of airway obstruction. The first because the effect of backward (bronchial-alveolar) NO diffusion decrease as the exhaled flow rate increase, consequently low flows tend to overestimate CaNO; however, selection of extreme flows is less feasible for children. We preferred easy-to-perform exhalations between 50 and 150 mL/s; thus, we can draw conclusions for our method only. The second factor potentially affecting alveolar NO measurements is peripheral airway obstruction; in this case however, CaNO is underestimated (rather than overestimated) when corrections for backward NO diffusion are applied.<sup>33</sup> None of the preceding factors was apparently relevant in our casistic, particularly because both corrected and uncorrected CaNO predicted EIB.

No studies have measured adenosine in the urine of steroid-naïve atopic asthmatic children. Adenosine concentrations in EBC have been found higher in asthmatic patients than in healthy controls,<sup>34,35</sup> being these concentrations still higher in patients with symptoms than in patients without symptoms.<sup>35</sup> In keeping, adenosine

concentrations in urine were found elevated in our atopic asthmatic patients as compared with their healthy controls; in addition, patients who develop EIB had higher baseline adenosine concentrations and reported more frequently postexercise cough than patients without EIB, suggesting poor disease control.

There is evidence that adenosine concentrations in plasma and EBC increase during exercise in atopic asthmatic patients with EIB but not in patients without EIB or control subjects.<sup>36,37</sup> Bronchial challenge with inhaled adenosine induces bronchoconstriction in asthmatic subjects but not in those healthy,<sup>38</sup> suggesting a previous adenosine-induced upregulation of the Th2 response.<sup>9</sup> Consistently with these findings, a possible role for adenosine in this asthma phenotype has been proposed.<sup>38</sup> The wide distribution and the complex interrelation of ATP and adenosine in several body tissues including the lung, have been recently summarized.<sup>39,40</sup> Because the production of both adenosine and exhaled NO share the Th2-cell-driven pathway, analyses for these substances to assess EIB could be only applicable in atopic patients.

ATP and adenosine are endogenous signaling molecules which closely interplay in inflammation and immunity to drive immune responses, either pro-inflammatory or downregulating the airway injury.<sup>9</sup> Purinergic-membrane receptors for ATP (7 P2x and 8 P2y subunits) and four receptors for adenosine (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>) are present in virtually all immunity cells.<sup>9,39</sup> Through several of these receptors both adenosine and ATP can induce mast cell degranulation by alone or enhancing the allergen-induced degranulation with the consequent release of histamine and inflammatory mediators.<sup>39</sup>

We used a known indirect airway challenge, i., exercise, whose mechanism involves hyperosmotic damage of airway-epithelial cells during hyperventilation with subsequent release of inflammatory mediators and bronchial hyperreactivity.<sup>26</sup> Our atopic patients with EIB had elevated adenosine levels yet before their exercise challenge suggesting current inflammation; in these conditions, adenosine acts as a feedback signaling for immunosuppression and tissue repair.<sup>9</sup> Elevated excretion of adenosine in urine could derive from its increased production in the asthmatic airways, as found in EBC.<sup>34,35</sup> This hypothesis could be better assessed by measuring adenosine in both urine and EBC before the exercise challenge. Further studies should better establish the source and the meaning of baseline urinary adenosine in patients prone to EIB.

We could not find differences for urinary 8-OxodG levels on depending of EIB in our patients, nor these levels distinguished patients from healthy controls. Previously we have demonstrated a relationship between high baseline concentrations of 8-Isoprostane in EBC and the postexercise fall in FEV<sub>1</sub> in asthmatic children and adolescents.<sup>7</sup> High levels of 8-Isoprostane and 8-OxodG reflect oxidant stress on different cellular targets; the former on cell membrane phospholipids, the latter on DNA.<sup>7,11</sup> Urinary excretion of 8-OxodG represents oxidative damage on guanine moieties of both DNA and its precursor pools, which is thought to be pivotal in several diseases.<sup>11</sup> Our study does not support urinary 8-OxodG as a biomarker of asthma and airway responsiveness. Nonetheless, this

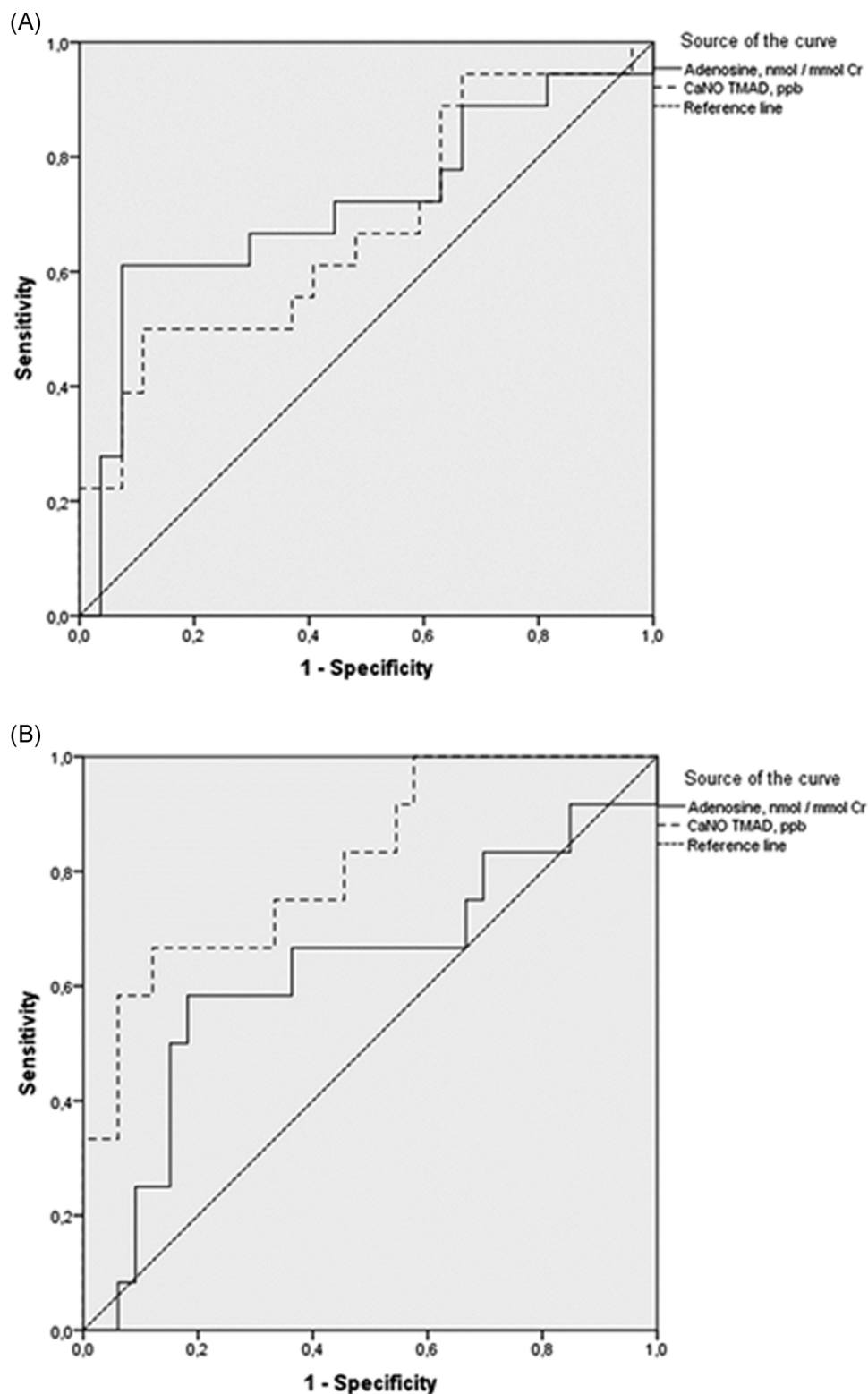


**FIGURE 2** Receiver operating characteristic (ROC) curves for normalized concentrations of urinary adenosine by urinary creatinine (nmol / mmol Cr) and alveolar nitric oxide corrected for the Trumpet Model of Airway Diffusion (CaNO TMAD, ppb) as predictors of EIB using two different criteria. A, Fall in FEV<sub>1</sub> ≥ 10%; areas under curves (AUCs): urinary adenosine: 0.71,  $P = .016$ ; CaNO TMAD: 0.68,  $P = .046$ . B, Fall in FEV<sub>25-75</sub> ≥ 26%; AUCs: urinary adenosine: 0.63,  $P = .191$ ; CaNO TMAD: 0.82,  $P = .001$ . See also predictive values for EIB by percentiles of each biomarker in Table 4. EIB, exercise-induced bronchoconstriction; TMAD, trumpet model of airway diffusion

and other urinary nucleosides should be tested before excluding oxidative injury on DNA from mechanisms of EIB.

In conclusion, we found that increased concentrations of two noninvasive (exhaled and urinary) inflammatory biomarkers predicted EIB in atopic asthmatic children. Measuring concentrations of the peripheral airway NO and urinary adenosine appear suitable for monitoring these patients; the measurement of adenosine in urine could become clinically useful when its analysis in real-time will be available. New studies are required to identify appropriate non-invasive biomarkers for assessing EIB in pediatric patients with other asthma phenotypes.

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## REFERENCES

- Cabral ALB, Conceição GM, Fonseca-Guedes CHF, Martins MA. Exercise-induced bronchospasm in children: effects of asthma severity. *Am J Respir Crit Care Med*. 1999;159:1819-1823.
- Brannan JD, Koskela H, Anderson SD. Monitoring asthma therapy using indirect bronchial provocation tests. *Clin Respir J*. 2007;1:3-15.
- Barreto M, Zambardi R, Villa MP. Exhaled nitric oxide and other exhaled biomarkers in bronchial challenge with exercise in asthmatic children: current knowledge. *Paediatr Respir Rev*. 2015;16(1):68-74.
- Linkosalo L, Lehtimäki L, Holm K, Kaila M, Moilanen E. Relation of bronchial and alveolar nitric oxide to exercise-induced bronchoconstriction in atopic children and adolescents. *Pediatr Allergy Immunol*. 2012;23:360-366.
- Chinellato I, Piazza M, Peroni D, et al. Bronchial and alveolar nitric oxide in exercise-induced bronchoconstriction in asthmatic children. *Clinical & Experimental Allergy*. 2012;42:1190-1196.
- Carraro S, Corradi M, Zanconato S, et al. Exhaled breath condensate cysteinyl leukotrienes are increased in children with exercise-induced bronchoconstriction. *J Allergy Clin Immunol*. 2005;115:764-770.
- Barreto M, Villa MP, Olita C, Martella S, Ciabattini G, Montuschi P. 8- Isoprostane in exhaled breath condensate and exercise-induced bronchoconstriction in asthmatic children and adolescents. *Chest*. 2009;135:66-73.
- Erzurum SC, Gaston BM. Biomarkers in asthma: a better hope to better manage asthma. *Clin Chest Med*. 2012;33(3):459-471.
- Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther*. 2006;112(2):358-404.
- Cooke MS, Olinski R, Loft S. European standards committee on urinary (DNA) lesion analysis. Measurement and meaning of oxidatively modified DNA lesions in urine. *Cancer Epidemiol Biomarkers Prev*. 2008;17(1):3-14.
- Poulsen HE, Nadal LL, Broedbaek K, Nielsen PE, Weimann A. Detection and interpretation of 8-oxodG and 8-oxoGua in urine, plasma and cerebrospinal fluid. *Biochim Biophys Acta*. 2014;1840(2):801-808.
- Vidotto C, Fousert D, Akkermann M, Griesmacher A, Müller MM. Purine and pyrimidine metabolites in children's urine. *Clin Chim Acta*. 2003;335(1-2):27-32.
- Taniai H, Sumi S, Ito T, Ueta A, Ohkubo Y, Togari H. A simple quantitative assay for urinary adenosine using column-switching high-performance liquid chromatography. *Tohoku J Exp Med*. 2006;208(1):57-63.
- Protano C, Andreoli R, Mutti A, Petti S, Vitali M. Biomarkers of oxidative stress to nucleic acids: background levels and effects of body mass index and life-style factors in an urban paediatric population. *Sci Total Environ*. 2014;500-501:44-51.
- Garratt LW, Mistry V, Singh R, et al. ARESTCF. Interpretation of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine is adversely affected by methodological inaccuracies when using a commercial ELISA. *Free Radic Biol Med*. 2010;48(11):1460-1464.
- Andreoli R, Spataro G, Pignini D, et al. Urinary biomarkers of exposure and of oxidative damage in children exposed to low airborne concentrations of benzene. *Environ Res*. 2015;142:264-272.
- Topic A, Francuski D, Nikolic A, et al. The role of oxidative stress in the clinical manifestations of childhood asthma. *Fetal Pediatr Pathol*. 2017;36(4):294-303.
- Global initiative for asthma. Global strategy for asthma management and prevention (2018 update). Available from: [www.ginasthma.org](http://www.ginasthma.org)
- Barreto M, Piacentini G, Chiossi L, et al. Tidal-breathing measurement of exhaled breath temperature (EBT) in schoolchildren. *Pediatr Pulmonol*. 2014;49(12):1196-1204.
- Tripodi S, Barreto M, Di Rienzo-Busincio A, et al. Asthma control test and bronchial challenge with exercise in pediatric asthma. *Front Pediatr*. 2016;4:16. <https://doi.org/10.3389/fped.2016.00016> Mar 8.
- American Thoracic Society. European Respiratory Society. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med*. 2005;171(8):912-930.
- Tsoukias NM, George SC. A two-compartment model of pulmonary nitric oxide exchange dynamics. *J Appl Physiol*. 1998;85(2):653-666.
- Condorelli P, Shin HW, Aledia AS, Silkoff PE, George SC. A simple technique to characterize proximal and peripheral nitric oxide exchange using constant flow exhalations and an axial diffusion model. *J Appl Physiol*. 2007;102(1):417-425.
- American thoracic society. Standardization of spirometry: 1994 update. *Am J Respir Crit Care Med*. 1995;152:1107-1136.
- Quanjer PH, Stocks J, Polgar G, et al. Compilation of reference values for lung function measurements in children. *Eur Respir J Suppl*. 1989;4:184S-261S.
- Crapo RO, Casaburi R, Coates AL, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med*. 2000 Jan;161(1):309-329.
- Custovic A, Arifhodzic N, Robinson A, Woodcock A. Exercise testing revisited: the response to exercise in normal and atopic children. *Chest*. 1994;105:1127-1132.
- Koh YI, Choi S. Blood eosinophil counts for the prediction of the severity of exercise-induced bronchospasm in asthma. *Respir Med*. 2002;96(2):120-125.
- Lex C, Dymek S, Heying R, Kovacevic A, Kramm CM, Schuster A. Value of surrogate tests to predict exercise-induced bronchoconstriction in atopic childhood asthma. *Pediatr Pulmonol*. 2007;42(3):225-230.
- Scollo M, Zanconato S, Ongaro R, Zaramella C, Zacchello F, Baraldi E. Exhaled nitric oxide and exercise-induced bronchoconstriction in asthmatic children. *Am J Respir Crit Care Med*. 2000;161(3 Pt 1):1047-1050.

31. Terada A, Fujisawa T, Togashi K, et al. Exhaled nitric oxide decreases during exercise-induced bronchoconstriction in children with asthma. *Am J Respir Crit Care Med*. 2001;164(10 Pt 1):1879-1884.
32. Nishio K, Odajima H, Motomura C, Nakao F, Nishima S. Exhaled nitric oxide and exercise-induced bronchospasm assessed by FEV1, FEF25-75% in childhood asthma. *J Asthma*. 2007;44(6):475-478.
33. Verbanck S, Malinowski A, George S, Gelb AF, Vincken W, VanMuylem A. Bronchial and alveolar components of exhaled nitric oxide and their relationship. *Eur Respir J*. 2012;39(5):1258-1261.
34. Esther CR, Jr, Boysen G, Olsen BM, et al. Mass spectrometric analysis of biomarkers and dilution markers in exhaled breath condensate reveals elevated purines in asthma and cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2009;296(6):L987-L993.
35. Huszár E, Vass G, Vizi E, et al. Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. *Eur Respir J*. 2002;20(6):1393-1398.
36. Vizi E, Huszár E, Csoma Z, et al. Plasma adenosine concentration increases during exercise: a possible contributing factor in exercise-induced bronchoconstriction in asthma. *J Allergy Clin Immunol*. 2002;109(3):446-448.
37. Csoma Z, Huszár E, Vizi E, et al. Adenosine level in exhaled breath increases during exercise-induced bronchoconstriction. *Eur Respir J*. 2005;25(5):873-878.
38. Fozard JR. The case for a role for adenosine in asthma: almost convincing? *Curr Opin Pharmacol*. 2003;3(3):264-269.
39. Gao ZG, Jacobson KA. Purinergic signaling in mast cell degranulation and asthma. *Front Pharmacol*. 2017;8:947. <https://doi.org/10.3389/fphar.2017.00947>.
40. Pelleg A, Schulman ES, Barnes PJ. Extracellular adenosine 5'-triphosphate in obstructive airway diseases. *Chest*. 2016;150(4):908-915.

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