

## ORIGINAL PAPER

# The effect of allergic rhinitis on adenosine concentration in exhaled breath condensate

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## Clinical and Experimental Allergy

### Summary

**Background** Patients with allergic rhinitis (AR) frequently develop asthma. This initiating inflammation in the lower airways may result in increased levels of inflammatory mediators such as adenosine in the exhaled breath.

**Objective** We compared adenosine levels in exhaled breath condensate (EBC) and both exhaled and nasal nitric oxide (NO) levels of AR patients and healthy control subjects. We also tested whether inhalation through inflamed nasal cavity during EBC sampling influences adenosine concentrations in exhaled air.

**Methods** Exhaled and nasal NO levels were measured and EBC samples (at oral inhalation) were collected from 27 patients and 15 healthy controls. EBC collection was repeated after 15 min with subjects inhaling through their nose. Adenosine was measured by HPLC and NO was determined by chemiluminescence.

**Results** The concentration of EBC adenosine was higher in patients with AR than in healthy controls ( $12.4 \pm 1.3$  nM vs.  $6.5 \pm 0.7$  nM,  $P = 0.0019$ ) and this was accompanied by an increase in the concentration of exhaled NO ( $10.2 \pm 1.3$  ppb vs.  $5.3 \pm 0.5$  ppb;  $P = 0.0099$ , respectively). No difference in nasal NO was detected. EBC adenosine concentration showed a significant positive correlation with the level of exhaled NO. In contrast to healthy control subjects, patients with rhinitis had higher levels of exhaled adenosine when inhaling via the nose instead of the mouth ( $17.7 \pm 2.8$  nM,  $P = 0.007$ ).

**Conclusion** When compared with healthy subjects, patients with AR exhibit an increased concentration of exhaled adenosine and a related increase in exhaled NO concentration. EBC adenosine is further increased when rhinitis patients inhale through their nose than via their mouth. Our data suggest that non-asthmatic patients with rhinitis may have subclinical inflammation in their lower airways.

**Keywords** adenosine, asthma, exhaled breath condensate, inflammation, nitric oxide, rhinitis  
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### Introduction

An increasing number of pathophysiological, epidemiological and clinical data suggest a link between allergic inflammation of the upper and lower airways [1–6], although the pathophysiology is still unclear. One of the possible explanations for the co-morbidity of asthma and rhinitis is that secretions from the inflamed nasal cavities enter the lower airways via aspiration or inhalation. Inflammatory mediators and cells from the nasal secretion may act as a trigger in the development of lower airway inflammation and, ultimately, asthma [7, 8].

Non-invasive methods such as the measurement of exhaled nitric oxide (NO) levels and analysis of exhaled breath condensate (EBC) have the potential to reveal inflammatory processes in the lower airways [9, 10]. The level of exhaled NO, a marker of eosinophil inflammation in asthma [11, 12], is increased in asymptomatic atopic subjects with subclinical airway inflammation [13] and also in patients with allergic rhinitis (AR) who have no symptoms of lower airway inflammation [14–16]. Although exhaled NO has been studied more extensively in patients with rhinitis, the analysis of EBC in this patient group has been very limited. Niimi et al. [17] detected

lower pH in the EBC of patients with AR than in healthy controls. Cap et al. [18] described higher than normal levels of leukotriene B4 and E4 in seasonal AR patients.

Adenosine has several proinflammatory effects [19]. Among others, it recruits neutrophils, causes bronchoconstriction and increases blood flow. Mice lacking adenosine deaminase, an enzyme that degrades adenosine, exhibit asthma-like phenotypes such as extensive mast cell degranulation, eosinophilia, elevated immunoglobulin (IgE) and an increased hyper-responsiveness accompanied by an elevated level of adenosine in the lungs [20, 21]. Elevated adenosine concentrations in serum [22, 23], in bronchoalveolar lavage [24] and in EBC [25] have been reported in asthmatic patients. Although its function in asthma is still unclear, these observations indicate an important role for adenosine in asthma [26]. On the basis of the pathophysiological similarities of asthmatic and rhinitic inflammation, it is likely that adenosine plays a role in the allergic inflammation of the upper airways as well. This concept is supported by our previous findings showing that a high level of adenosine is present in the nasal lavage of AR patients [27].

Adenosine can be reproducibly measured in EBC [25, 27]. The level of exhaled adenosine is increased in asthma and correlates well with exhaled NO levels [25], suggesting that EBC adenosine reflects the intensity of inflammation in the lower airways. The aim of our current study was to assess the level of EBC adenosine and to correlate that to exhaled NO levels in patients with non-asthmatic AR. We also investigated whether inhalation via the inflamed nasal cavities during EBC sampling influences the level of EBC adenosine in these patients.

## Methods

### Subjects

A total of 27 patients with AR and 15 healthy volunteers with no history of chronic diseases or airway infections in the previous 2 weeks were studied (Table 1). All patients had symptoms of rhinitis at the time of measurement but none of them had a history of asthma or asthmatic symptoms. All rhinitis patients had persistent disease (symptoms were present for more than 4 weeks and at least 4 days a week) [7]. Six patients were treated with nasal steroid and 21 of them were steroid-naïve. Atopic status of patients with rhinitis was checked by skin prick

test. All patients showed positive test results to at least one of the following common aeroallergens: *Abrosia artemisiifolia*, *Artemisia vulgaris*, *Lolium perenne*, *Betula pendula*, *Aspergillus fumigatus*, *Dermatophagoides pteronyssinus* and *Felix domesticus* (data not shown). All participants except three patients with AR were never-smokers. The three ex-smokers had no elevated exhaled carbon monoxide levels at the time of the study. Because they were not heavy smokers (less than one package-year in their smoking history), and they stopped smoking at least 3 years before the examination, they were not excluded from the study.

Smoking habit was tested by NicCheck I (DynaGen Inc., Cambridge, MA, USA) [28]. The local Ethical Committee approved the protocol, and informed consent was obtained from each subject.

### Study design

After measuring exhaled and nasal NO levels, EBC was collected for 10 min during tidal breathing while subjects were wearing a nose clip (inhalation through the mouth). Following a 15-min break, EBC collection was repeated without a nose clip in 23 rhinitis patients (17 steroid-naïve) and 15 healthy controls. Subjects inhaled through their nose and then exhaled via the mouth (inhalation through the nose). Our previous study showed that the order of these two different collection modes had no effect on adenosine levels [27]. Samples were stored at  $-70^{\circ}\text{C}$  for further analysis. After EBC sampling, a lung function test was performed.

### Exhaled and nasal nitric oxide measurement

Exhaled and nasal NO levels were measured by a chemiluminescence analyzer (Model LR2000; Logan Research, Rochester, UK) according to international guidelines [15, 29]. The analyzer is sensitive to NO from 1–5000 parts per billion (ppb) by volume with a resolution of 0.3 ppb. It was designed for online recording of exhaled and nasal NO concentrations. Calibration was performed with a certified NO mixture (90 ppb) in nitrogen (BOC Special Gases, Guildford, UK). To prevent nasal contamination, measurement of exhaled NO was made by slow exhalation (5 L/min) from total lung capacity for  $20 \pm 30$  s against a resistance ( $0.4 \pm 0.05$  kPa, or  $3.0 \pm 0.4$  mmHg). Nasal NO level was measured during breathholding (soft palate closed) with a probe inserted into one of the nares [30].

### Exhaled breath condensate collection

EBC was collected using a condenser (EcoScreen, Jaeger, Hoechberg, Germany) as previously published [27]. Briefly, subjects breathed through a mouthpiece and a two-way non-rebreathing valve, which served as a saliva

Table 1. Subject characteristics

|                                | Healthy subjects | Patients with allergic rhinitis |
|--------------------------------|------------------|---------------------------------|
| N                              | 15               | 27                              |
| Age (year)                     | $25.7 \pm 1.2$   | $35.0 \pm 2.4$                  |
| Female/male                    | 6/9              | 18/9                            |
| FEV <sub>1</sub> (% predicted) | $96.3 \pm 2.4$   | $97.2 \pm 2.3$                  |

trap. Subjects were asked to breathe at a normal frequency and tidal volume, with or without a nose-clip (as detailed above), for a period of 10 min. Mean expiratory flow rates were between 205 and 330 mL/s. EBC samples were centrifuged and stored at  $-70^{\circ}\text{C}$  until adenosine measurements.

### Lung function test

FEV<sub>1</sub> was measured according to the American Thoracic Society guidelines [31] using an electronic spirometer (MEDICOR MS-11; Piston Ltd, Budapest, Hungary).

### Adenosine measurement

Adenosine concentrations were determined by HPLC (Pharmacia LKB; HPLC System, Uppsala, Sweden) [25, 32]. Intra-assay CV of the HPLC method is under 10%. The detection limit is 2 nM.

### Statistical analysis

Because data were normally distributed, parametric tests were used for statistical analysis. Differences between healthy and the rhinitis group were assessed by unpaired *t*-test, and between the two collection methods by paired *t*-test (GraphPad Prism; GraphPad Software, Inc., San Diego, CA, USA). Data are expressed as mean  $\pm$  SEM, and significance defined as  $P < 0.05$ .

## Results

### Exhaled nitric oxide and exhaled breath condensate adenosine in patients with allergic rhinitis

Exhaled NO levels were significantly higher in patients with AR as compared with healthy controls ( $10.2 \pm 1.3$  ppb vs.  $5.3 \pm 0.5$  ppb, respectively,  $P < 0.0099$ ; Fig. 1a). Adenosine concentration in EBC (obtained by oral inhalation) was significantly higher in patients with AR than in healthy controls ( $12.4 \pm 1.3$  nM vs.  $6.5 \pm 0.7$  nM, respectively,  $P = 0.0019$ ; Fig. 1b). Nasal NO level was

$880 \pm 72$  ppb in rhinitis patients, and  $772 \pm 77$  ppb in healthy volunteers, with no significant difference between the two groups (Fig. 1c). No difference was seen in any of these parameters between steroid-naïve ( $n = 21$ ) and steroid-treated ( $n = 6$ ), or between male ( $n = 9$ ) and female ( $n = 18$ ) patients with rhinitis (data not shown).

The level of EBC adenosine showed significant correlation with exhaled NO ( $P = 0.007$ ,  $r = 0.41$ ,  $n = 42$ ; Fig. 2), but not with nasal NO. Interestingly, the volume of EBC samples of patients with rhinitis was significantly smaller than those of healthy subjects ( $1442 \pm 91$   $\mu\text{L}$  vs.  $1768 \pm 75$   $\mu\text{L}$ ,  $P = 0.019$ , respectively). Normalization of adenosine levels to the volume calculated by multiplying the adenosine concentration by the volume of the same sample (= absolute values) did not change the difference between the groups (not shown).

### Effect of nasal inhalation on exhaled breath condensate adenosine levels in allergic rhinitis patients

The mode of inhalation during EBC collection had no influence on exhaled adenosine levels in healthy subjects ( $6.5 \pm 0.7$  nM by oral inhalation,  $6.8 \pm 0.8$  nM by nasal inhalation; Fig. 3, left panel). In contrast, EBC samples from patients with AR had, on average, a 50% higher level of adenosine when, during sampling, patients inhaled via the nose as opposed to the mouth ( $17.7 \pm 2.8$  nM vs.  $12.0 \pm 1.4$  nM, respectively,  $P = 0.006$ ; Fig. 3, right panel). Again, there was no difference between steroid-naïve and steroid-treated patients. The volume of EBC was higher for both groups when subjects inhaled via the nose rather than the mouth (healthy:  $2160 \pm 146$   $\mu\text{L}$  vs.  $1768 \pm 75$   $\mu\text{L}$ ,  $P = 0.014$ ; AR:  $1676 \pm 118$   $\mu\text{L}$  vs.  $1442 \pm 91$   $\mu\text{L}$ ,  $P = 0.02$ , respectively). Here again, calculating with volume-normalized adenosine data did not change the outcome (not shown).

## Discussion

In the present study, we set out to look for the presence of a potential subclinical inflammation in the lower airways

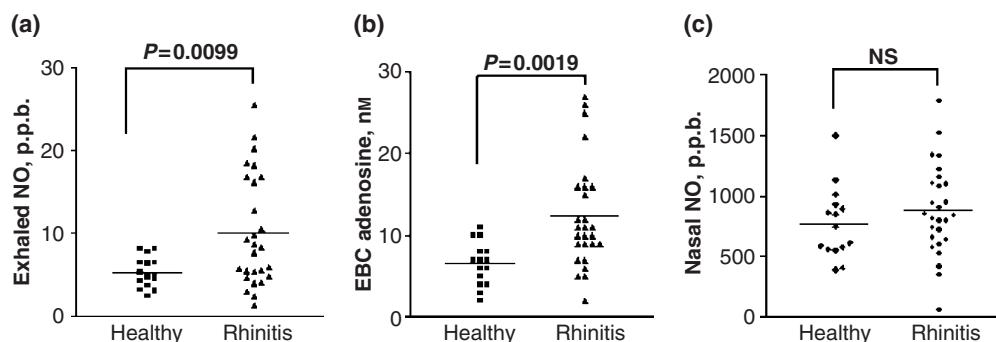


Fig. 1. Individual exhaled nitric oxide (a), exhaled breath condensate adenosine (b) and nasal nitric oxide (c) levels of healthy subjects ( $n = 15$ ) and allergic rhinitis patients ( $n = 27$ ).

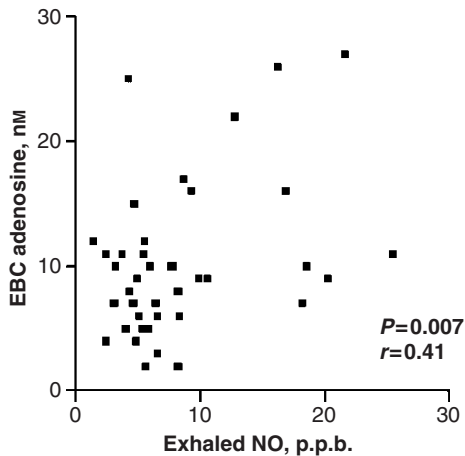


Fig. 2. The relation between exhaled nitric oxide and exhaled breath condensate adenosine concentrations in participating subjects ( $n = 42$ ).

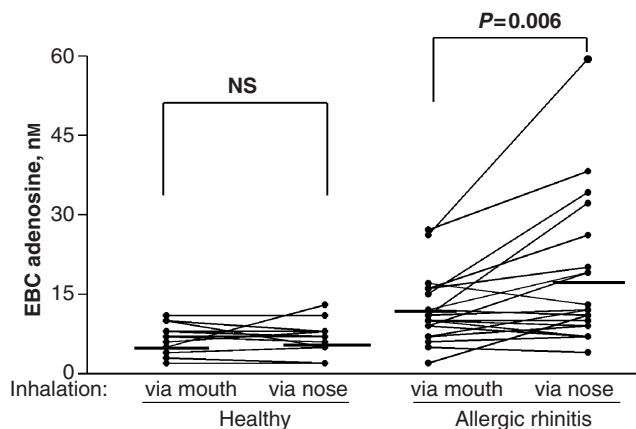


Fig. 3. Exhaled breath condensate was collected from 23 allergic rhinitis patients (right panel) and 15 healthy control subjects (left panel) in two consecutive samplings: first, while wearing a nose clip, they inhaled and exhaled via the mouth and, second, without a nose clip, they inhaled through their nose and exhaled via the mouth. Horizontal lines indicate the mean values for each group.

of patients with AR. We have shown that, in pollen season, the level of adenosine in EBC is elevated in non-asthmatic AR patients as compared with healthy control subjects. Adenosine concentration correlates well with the concentration of exhaled NO, an accepted marker of lower airway inflammation [33]. Adenosine concentration was further elevated in patients with rhinitis when EBC was collected while patients were breathing through their nose rather than their mouth. There was no such increase in healthy control subjects.

Exhaled NO levels were increased in our patients with AR as compared with healthy controls. This finding is in the main line of previous studies [34–36]. There are only few publications not detecting elevated exhaled NO in patients with rhinitis, either because different technique

was used to measure NO in exhaled breath [37], or because of the lack of atopic status that seems to be crucial for finding elevated exhaled NO in patients with rhinitis [38–40]. In our study, all patients were atopic to at least one common allergen. Because an increase in the concentration of exhaled NO is a sign of inflammation of the lower airways, these data confirm previous suggestions that (some) patients with AR have a clinically non-manifested lower airway inflammation.

This concept is further supported by our observation that the level of adenosine in EBC was also elevated in patients with AR. Adenosine is unquestionably involved in the pathophysiology of asthma as demonstrated by *in vitro* and murine studies [19–21]. Previous data also showed that adenosine concentration is enhanced in bronchoalveolar lavage and EBC of patients with asthma, implying that adenosine is an indicator of asthmatic inflammation. Because EBC is believed to sample the lung, we think that the increased adenosine level in these patients reflects inflammatory changes of the lower airways. This hypothesis is further supported by the fact that adenosine concentrations in rhinitis patients were in the same range as found previously in asthmatic patients [25, 41], and that adenosine levels correlated significantly with another inflammatory marker, exhaled NO. Although the difference in age and sex proportion between the patient and healthy groups as potential confounding factors cannot be ruled out, our data suggest that elevated levels of exhaled adenosine in patients with rhinitis indicate an existing inflammation of the lower airways. It is worthy to consider close monitoring of these patients for early signs of asthma. Measuring the level of exhaled adenosine together with NO could be a way to non-invasively screen these patients.

Both EBC adenosine and exhaled NO showed a marked overlap between patients and healthy control subjects. This is not surprising, nor does it contradict our concept of an existing subclinical inflammation in the lung, because asthma does not develop in all, but only about 30% of patients with rhinitis. Interestingly, about one-third of our rhinitis patients had a higher level of exhaled NO than the highest level within the healthy control group. A similar ration was found for EBC adenosine.

We found no association of exhaled adenosine levels with either nasal steroid treatment or the gender of patients with rhinitis.

Unlike the consistently observed increase of exhaled NO in AR patients, there are conflicting results on nasal NO levels. Some studies reported an increase in nasal NO levels [14, 15, 42], whereas others – similar to our findings here – found no difference in comparison with healthy controls [43–45]. Because NO is a marker of inflammation, we would expect elevated nasal NO levels in rhinitis. One of the possible explanations for these discrepancies might be that it is not only the amount of NO production that

influences the detected NO levels, but also the passage of NO from the production sites (mostly paranasal sinuses) to the nasal cavity where it is sampled. In another words, if something (such as swollen nasal mucosa, secretions or polyps) blocks the ostiae of sinuses, it can result in reduction of measured nasal NO, even though the production might be increased [46]. Contribution of other factors such as methodological differences or the cross-sectional nature of studies cannot be ruled out either.

In our previous study, we showed that nasal inhalation during EBC collection does not influence the content of exhaled air as compared with oral inhalation [27]. In the second part of our recent study, we carried out a similar comparison in patients with rhinitis who have evident inflammation in their nose. We determined the level of EBC adenosine in samples collected by nasal and oral inhalation. The rationale is looking for potential changes of this particular marker was that patients with AR exhibited high levels of adenosine in their nasal cavities. Interestingly, unlike in healthy subjects, EBC adenosine was significantly increased in rhinitis patients when they inhaled through their nose during sample collection as compared with oral inhalation. The increased EBC adenosine level by nasal inhalation in rhinitis patients can be explained by the high adenosine levels of the inflamed upper airways, and suggests that part of the exhaled adenosine originates from the nose. This explanation is consistent with reports postulating the presence of a 'chronic circulation' of inflammatory mediators from the upper into the lower airways, ultimately contributing to the development of asthma [1, 7, 8, 47, 48].

Despite several advantages of not using a nose clip during sampling [27], the preferred way of condensate collection is that subjects wear a nose clip during sampling, so that respiration is restricted to the oral cavity and nasal contamination of the condensate is limited to a minimum [49]. Our present study makes a very good case for consistently using a nose clip during EBC collection.

In summary, our study supports the concept of one airway-one disease. We showed that non-asthmatic patients with AR have elevated levels of exhaled NO and adenosine, two markers of inflammation, indicating the presence of a clinically non-manifested inflammation in the lower airways. Interestingly, inhalation via nose in patients with rhinitis increases EBC adenosine levels, unlike in healthy subjects where the mode of inhalation does not influence EBC content.

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