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Increased urinary leukotriene E_4 concentration in patients with eosinophilic pneumonia

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ABSTRACT: Although eosinophils produce cysteinyl leukotrienes (CysLTs) in large quantities, information on the relationship between CysLTs and eosinophilic pneumonia (EP) is lacking. Inflammatory mediator concentrations in urine were quantified to clarify the relationship between CysLT concentrations and EP severity.

Leukotriene (LT)E_4, eosinophil-derived neurotoxin (EDN), 9α,11β-prostaglandin F2 and LTB_4 glucuronide concentrations were quantified in the urine of: EP patients during acute exacerbation and clinical remission; asthmatic patients during acute exacerbation and under stable conditions; and healthy control subjects.

The urinary LTE_4 and EDN concentrations of EP patients during acute exacerbation were significantly higher than those of asthmatic patients and healthy subjects, and decreased immediately during clinical remission. The urinary LTE_4 concentration was associated with the urinary EDN concentration of EP patients during acute exacerbation. The urinary LTE_4 concentration significantly correlated with the diffusing capacity of the lung for carbon monoxide in EP patients during acute exacerbation.

The increased urinary concentrations of leukotriene and eosinophil-derived neurotoxin were associated with acute exacerbation in eosinophilic pneumonia patients. The increased leukotriene concentration significantly correlated with diffusing capacity of the lung for carbon monoxide, suggesting that the monitoring of leukotriene concentration may aid in the management of eosinophilic pneumonia patients.

KEYWORDS: Biomarkers, bronchial asthma, diffusing capacity, eicosanoids, eosinophilic pneumonia, leukotriene

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STATEMENT OF INTEREST
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Eosinophilic pneumonia (EP) is a diffuse infiltrative lung disease characterised by alveolar and peripheral airway eosinophilia [1–4]. Idiopathic EP is divided into two clinical entities: acute EP (AEP) and chronic EP (CEP). AEP shows good response to corticosteroid therapy and does not generally progress to CEP. Although corticosteroids are effective in almost all EP patients, the relapse rate during corticosteroid tapering is very high.

Although the mechanisms of eosinophilic accumulation remain to be elucidated, increasing evidence suggests the important roles of cytokine, chemokine and lipid mediators in the regulation of eosinophilic inflammation in various eosinophilic airway diseases [5]. On the basis of studies of bronchoalveolar lavage fluid (BALF) from EP patients, the concentrations of prostaglandin (PG)E_2, interleukin (IL)-5, RANTES (regulated on activation, normal T-cell expressed and secreted), eotaxin and monocyte chemotactic protein-1, which are all potent stimulators of eosinophils through their activation, degranulation and inhibition of apoptosis [6], were found to be significantly increased [1, 7, 8]. The essential components of eosinophil migration into the lung, such as leukotriene (LT)D_4, LTB_4 and IL-5, are generally considered chemotactic factors. In other inflammatory diseases associated with eosinophilia, such as allergic asthma, aspirin-intolerant asthma and nasal polyposis, local eosinophil accumulation closely correlates with tissue cysteinyl LT (CysLT) concentration [9, 10]. Although eosinophils have the capacity to generate LTC_4 in large quantities, to date there are no reports on the involvement of CysLTs in EP patients.

Urine has been found to be a useful biological fluid for monitoring the endogenous release of

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inflammatory mediators. The urinary metabolite concentration, which can be easily determined, can be used to monitor the whole-body production of the precursor. The urinary LTE_4 concentration is considered a good marker of LTC_4 production in the human body. Similarly, LTB_4 glucuronides have been used as a marker for the whole-body production of LTB_4 [11]. PGD_2 has been identified as the main metabolite of arachidonic acid metabolism by the cyclooxygenase pathway in mast cells. After generation in the body, PGD_2 is degraded to 9α,11β-prostaglandin F2 (9α,11β-PGF2), which is subsequently excreted into the urine. Urinary 9α,11β-PGF2 is presumably related to mast-cell activation [12]. Eosinophil-derived neurotoxin (EDN) is used as an eosinophil degranulation marker in urine. EDN is released from eosinophil granules together with eosinophil cationic proteins and eosinophil peroxidise (EPO). The molecular weight of EDN is 18–19 kDa, which signifies that EDN is excreted in the urine more easily than EPO, which has a molecular weight of 66 kDa.

The aim of the present study was to evaluate the urinary inflammatory mediator concentrations in EP patients, and thereby clarify the relationship between LT and EP pathogenesis. The current study is the first report showing that the systemic production of CysLTs is elevated in EP patients during acute exacerbation.

METHODS

Subjects

The present study hospital-based study was conducted from January 2004 to December 2006. The diagnosis of EP was established on the basis of currently used criteria and the patients were classified as having AEP or CEP according to their clinical–radiological presentation [1, 3, 4]. All four of the following criteria had to be met by the patient in order to be included: 1) pulmonary infiltrates predominantly affecting the periphery of the lung on chest imaging; 2) blood and alveolar eosinophilia (based on the currently used criteria); 3) general and respiratory symptoms for >2 weeks; and 4) exclusion of known causes of EP (particularly drugs, parasitic infection, allergic bronchopulmonary mycosis and Churg–Strauss syndrome). Patients were excluded if they had signs of involvement outside the respiratory system compatible with Churg–Strauss syndrome and/or idiopathic hypereosinophilic syndrome. Patients who had taken medications such as LT receptor antagonists and oral corticosteroids prior to the study or patients who had exacerbated asthma for at ⩾3 months preceding the study were also excluded. For comparative analysis of urinary mediator data, 18 patients with bronchial asthma (BA) during acute exacerbation (BA-exacerbation group), 15 patients with BA under clinically stable condition (BA-stable group), and 15 healthy control subjects (HC group) were enrolled. The diagnosis of asthma was based on American Thoracic Society (ATS) criteria [13]. Asthma exacerbation was not only defined as episodes of shortness of breath, cough, wheezing, respiratory distress or a combination of these symptoms, but also a decrease in forced expiratory volume in one second of ⩾20% from the previous best values by measuring lung function [14]. The HC group was enrolled without any subjective symptoms or objective findings of diseases, including allergic diseases. All of the enrolled subjects confirmed they could tolerate nonsteroidal anti-inflammatory drugs on the basis of negative past history and/or aspirin provocation results. Permission to conduct the study was obtained from the Ethics Committee of the Sagamihara National Hospital (Kanagawa, Japan) and all the subjects gave their informed consent.

Study design

Urine samples were collected between 08:00 and 10:00 h from EP patients, the BA-stable group and the HC group. In particular, during the acute exacerbation of EP or acute asthmatic exacerbation, urine samples were collected before intensive corticosteroid therapy. To confirm the relationship between the clinical conditions and urinary LTE_4 concentrations in EP patients, the urinary LTE_4 concentrations were compared before and after therapy. In EP patients, the diffusing capacity of the lung for carbon monoxide ($D_{L,CO}$) was measured based on the ATS guidelines [15].

Measurements

Urine was collected in polypropylene bottles containing 4-hydroxy-TEMPO and the aliquots were stored at -35°C until analysis. LTE_4 was quantified using a commercial enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) after purification by HPLC as reported previously [16]. EDN was quantified using an EIA kit (MBL, Nagoya, Japan) after diluting the urine 50 times in PBS [17]. 9α,11β-PGF2 was quantified by EIA (Cayman Chemical) after extraction with an Empore C18 disk cartridge (3M, St Paul, MN, USA), according to the method reported by O'Sullivan *et al.* [18]. LTB_4 glucuronide (LTBG) concentration was determined from the LTB_4 concentration after hydrolysis with β-glucuronidase as reported previously [19]. The LTB_4 concentration was determined by EIA (Cayman Chemical). The concentrations of all the mediators were normalised to urinary creatinine (cr) concentration.

Statistical analysis

Data from the four groups (EP during acute exacerbation, BA-exacerbation, BA-stable and HC) were initially analysed by the Kruskal–Wallis H-test, a nonparametric statistical test. When the test showed a significant difference, pairwise comparisons were tested using the Mann–Whitney U-test with Bonferroni's correction (unpaired). Differences in the urinary biomarker concentrations of EP patients between exacerbation and clinical remission were evaluated using the Wilcoxon t-test. Relationships were analysed by the Spearman rank correlation test. A p-value <0.05 was regarded as statistically significant.

RESULTS

Among the 25 subjects who were approached to identify the sample population, six EP patients were excluded, of whom three had taken corticosteroids prior to the study, two had exacerbated asthma within 1 month preceding the study and one had suspected Churg–Strauss syndrome. In total, 19 idiopathic EP patients, consisting of two patients with AEP and 17 patients with CEP, were enrolled in the current study. The clinical characteristics of the EP patients are shown in table 1. Among the 17 CEP patients, eight had CEP accompanied by BA according to their past histories (CEP-BA) and nine had only CEP (CEP-alone). The asthma conditions of the patients in the CEP-BA group were clinically stable; four

patients had recurrent CEP. All the EP patients received systemic corticosteroids. After therapy, the clinical conditions of all the patients improved immediately.

Urinary LTE_4 concentration: comparison among EP patients, asthmatics and HCs

Figure 1 shows the urinary LTE_4 concentrations in all the groups. The urinary LTE_4 concentration was significantly higher in the EP patients during clinical exacerbation, including both AEP and CEP patients (median 719 $pg \cdot mg^{-1}$ cr), than in the BA-exacerbation group (138 $pg \cdot mg^{-1}$ cr; $p<0.001$), the BA-stable group (79 $pg \cdot mg^{-1}$ cr; $p<0.001$) and the HC group (66 $pg \cdot mg^{-1}$ cr; $p<0.001$).

Urinary EDN, 9α,11β-PGF2 and LTBG concentrations in EP patients

The urinary EDN, 9α,11β-PGF2, and LTBG concentrations in all the groups are shown in table 2. The urinary EDN concentration was significantly higher in the EP patients during clinical exacerbation (median 1,827 $ng \cdot mg^{-1}$ cr) than in the BA-exacerbation group (886 $ng \cdot mg\text{-}cr^{-1}$; $p=0.038$), BA-stable group (522 $ng \cdot mg^{-1}$ cr; $p=0.022$) and HC group (408 $ng \cdot mg^{-1}$ cr; $p=0.015$). However, there were no significant differences in the urinary 9α,11β-PGF2 concentration and LTBG concentration among the EP patients during clinical exacerbation, the BA-exacerbation group, BA-stable group and HC group. As the patients who participated in this study had normal renal, biliary or metabolic clearance, the concentrations of urinary metabolites were compared after correction for cr concentration. The concentrations of LTBG and 9α,11β-PGF2 were slightly lower than that of LTE_4. The concentrations of these metabolites were above the limit of detection of each EIA kit (Cayman Chemical). The limits of detection for the LTB_4 and 9α11β-PGF2 EIAs were 6 and 15 $pg \cdot mL^{-1}$, respectively. The median (range) concentrations of LTBG and 9α,1β-PGF2 were 10.2 (7.5–15.6) and 52 (27–382) $pg \cdot mL^{-1}$, respectively, without correction using the cr concentration.

Changes in urinary LTE_4 and EDN concentrations during the clinical disease course in CEP patients

The relationship between the concentrations of urinary mediators and the clinical disease course was examined in the 17 CEP patients (both the CEP-BA group and CEP-alone group). As shown in figure 2, the median (range) concentration of urinary LTE_4 in CEP patients significantly decreased from 823 (115–8,553) to 117 (19–530) $pg \cdot mg^{-1}$ cr ($p=0.003$) and that of EDN decreased from 1,827 (70–7,890) to 840 (20–51,999) $ng \cdot mg^{-1}$ cr ($p=0.021$). Data are expressed as the concentrations in clinical exacerbation *versus* those in clinical remission after the therapy. However, the urinary 9α,11β-PGF2 and LTBG concentrations did not change significantly during the clinical disease course in CEP patients. No significant difference was found in the concentrations of all the mediators measured in the present study between the asthmatics (CEP-BA) and nonasthmatics (CEP-alone).

Correlation between urinary LTE_4 concentration and EDN concentration in CEP patients

Following this, the correlation between the concentrations of the urinary mediators in CEP patients was examined. As shown in figure 3, there was a significant correlation between urinary LTE_4 concentration and EDN concentration during clinical exacerbation ($r=0.668$, $p=0.033$, $n=17$), but not during clinical remission after the therapy in CEP patients. However, no significant correlation between urinary LTE_4 concentration and 9α,11β-PGF2 concentration, urinary LTE_4 concentration and LTBG concentration or between the concentrations of any other urinary mediators was found.

Association between concentrations of urinary mediators and clinical characteristics of CEP patients

A significant correlation was found between the urinary LTE_4 concentration and 100 - $D_{L,CO}$ (% predicted) in pulmonary functional tests during clinical exacerbation of the disease in the 17 CEP patients ($r=0.788$, $p=0.002$; fig. 4). There was no significant correlation between the concentrations of any other

TABLE 1 Characteristics of patients with eosinophilic pneumonia

	AEP	CEP alone	CEP+BA
Male/female n	0/2	5/4	2/6
Age yr	19 (18–20)	52 (31–66)	55 (40–74)
Smoker/ex-smoker/nonsmoker n	2/0/0	2/3/4	0/2/6
White blood cells $\times 10^6 \cdot L^{-1}$	7220 (4500–9500)	12580 (8200–20100)*	11620 (6000–15300)*
Blood eosinophils %	14 (12–16)	26 (11–71)	30 (12–54)
CRP $mg \cdot mL^{-1}$	8.6	6.2	5.5
Total serum IgE $IU \cdot mL^{-1}$	31 (15–47)	172 (24–1240)*	312 (63–12500)*
BAL total cells $\times 10^5 \cdot mL^{-1}$	4.1 (3.5–4.6)	7.7 (2.5–12.0)	8.2 (3.1–15.6)
BAL eosinophils %	38 (35–41)	42 (28–52)	56 (27–73)
P_{a,O_2} Torr	86 (82–89)	78 (72–95)	77 (65–85)
FEV_1 % pred	89 (85–92)	86 (78–117)	78 (73–92)
$D_{L,CO}$ % pred	81 (75–86)	82 (65–92)	76 (55–88)

Data are presented as median (range), unless otherwise stated. AEP: acute eosinophilic pneumonia; CEP: chronic eosinophilic pneumonia; BA: bronchial asthma; CRP: C-reactive protein; Ig: immunoglobulin; BAL: bronchoalveolar lavage; P_{a,O_2}: arterial oxygen tension; FEV_1: forced expiratory volume in one second; % pred: % predicted; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide. *: $p<0.05$ compared with patients with AEP.

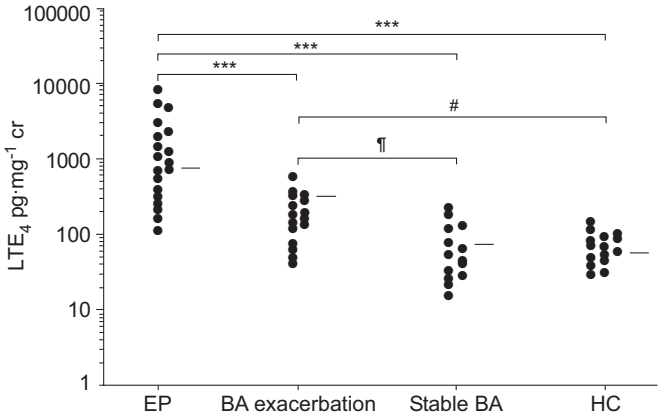

FIGURE 1. Urinary leukotriene (LT)E_4 concentrations in 19 eosinophilic pneumonia (EP) subjects, 18 patients with exacerbations of bronchial asthma (BA), 15 patients with stable BA and 15 healthy controls (HC). Horizontal bars indicate medians. cr: creatinine. #: p=0.036; ¶: p=0.042; ***: p<0.001.

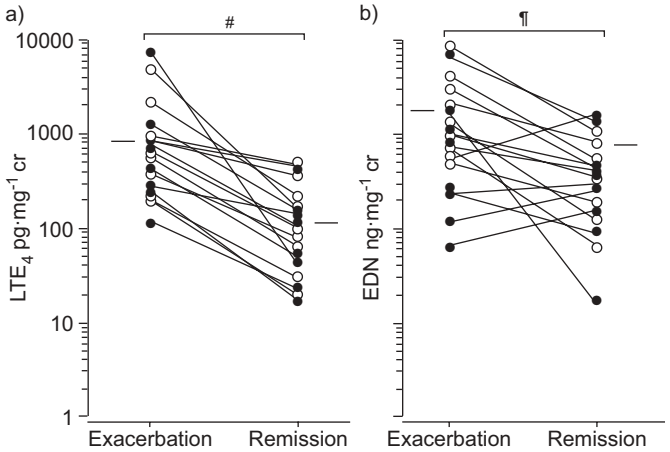

FIGURE 2. Changes in a) urinary leukotriene (LT)E_4 and b) eosinophil-derived neurotoxin (EDN) concentrations during exacerbations (pre-therapy) and remissions (post-therapy) in chronic eosinophilic pneumonia (CEP) patients. cr: creatinine. ○: CEP alone (n=9); ●: CEP plus bronchial asthma (n=8). Horizontal bars indicate medians. #: p-0.003; ¶: p=0.021.

urinary mediators measured in the present study and clinical characteristics of CEP patients. In addition, no correlation was found between the urinary LTE_4 concentration and the number of eosinophils in peripheral blood or BALF. No significant difference in clinical characteristics was found between the CEP-BA group and CEP-alone group.

DISCUSSION

In the current study, it was demonstrated for the first time that the urinary LTE_4 concentration was significantly higher in EP patients during acute exacerbation than in asthma patients with acute exacerbation and healthy subjects, and the concentration significantly decreased in EP patients during clinical remission. These findings suggest that CysLT production is closely associated with the clinical conditions of EP patients.

The urinary LTE_4 concentration is currently considered as one of the best available markers of *in vivo* CysLT production [20]. The urinary LTE_4 concentration increased in asthmatic patients during the early asthmatic response after allergen challenge [21], in exercise-induced asthma patients during bronchoconstriction [22] and in aspirin-intolerant asthma after aspirin provocation [18]. However, none of the EP patients who participated in the present study, despite their asthma complications, experienced an asthmatic exacerbation or showed any symptoms of airway narrowing. The current authors do not have a satisfactory explanation as to why the EP patients did not show impaired central bronchi function even if they showed eosinophil infiltration in the lungs similar to that observed in asthmatic patients [23, 24]. It may be possible that more mast cells infiltrate the bronchial smooth muscle of asthmatic patients and infiltrating mast cells may contribute to airway hyperresponsiveness and intermittent bronchoconstriction through the local release of inflammatory mediators, such as histamine, LTC_4 and PGD_2 in asthmatic patients [25]. Conversely, there is no sufficient evidence that mast cells infiltrate the bronchial smooth muscle of EP patients. The EP pathogenesis may be independent of mast cell activation because the 9α,11β-PGF2 concentrations did not increase during the exacerbation of the disease (table 2). It may be speculated that LTC_4 is produced by eosinophils in the lungs of EP patients, although the physiological stimuli that trigger eosinophils to generate LTC_4 have not been fully elucidated.

TABLE 2 Concentrations of urinary mediators

	AEP	CEP	CEP+BA	BA exacerbation	Stable BA	HC
Subjects n	2	9	8	18	15	15
LTE_4 pg·mg^{-1} cr	2502 (1952–3051)***,##,¶¶	722 (160–5520)***,##,¶¶	855 (115–8553)***,##,¶¶	138 (80–566)*,#	79 (50–221)	66 (34–178)
EDN ng·mg^{-1} cr	1150 (935–1365)*,#,¶	1850 (556–7890)*,#,¶	2312 (70–7582)*,#,¶	886 (135–1522)*,#	522 (152–885)	408 (180–620)
9α, 11β-PGF2 pg·mg^{-1} cr	108 (76–140)	85 (35–136)	72 (3–1067)	92 (25–520)	95 (5–202)	82 (7–195)
LTBG pg·mg^{-1} cr	12.4 (10.1–14.7)	7.2 (2.2–10.6)	6.5 (1.8–14.7)	7.5 (2.8–17.2)	7.1 (3.5–12.3)	6.7 (3.3–10.1)

Data are presented as median (range), unless otherwise stated. AEP: acute eosinophilic pneumonia; CEP: chronic eosinophilic pneumonia; BA: bronchial asthma; HC: healthy controls; LTE_4: leukotriene E_4; cr: creatinine; EDN: eosinophil-derived neurotoxin; 9α,11β-PGF2; 9α, 11β-prostaglandin F2; LTBG: leukotriene B_4 glucuronide.*: p<0.05 and ***: p<0.001 compared with HC group; #: p<0.05 and ##: p<0.001 compared with BA-stable group; ¶: p<0.05 and ¶¶: p<0.001 compared with BA-exacerbation group.

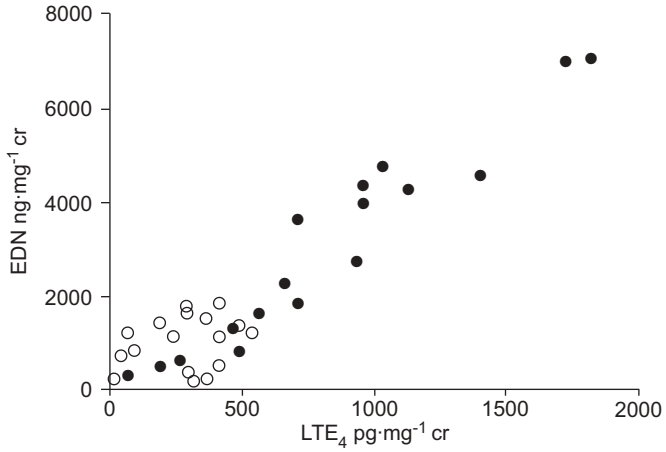

FIGURE 3. Correlation between urinary leukotriene (LT)E_4 and eosinophilic-derived neurotoxin (EDN) concentrations in chronic eosinophilic pneumonia patients. There was a significant correlation between urinary LTE_4 and EDN concentrations during clinical exacerbation (●: r=0.668, p=0.033, n=17), but not during clinical remission after therapy (○: r=0.255, p=0.379). cr: creatinine.

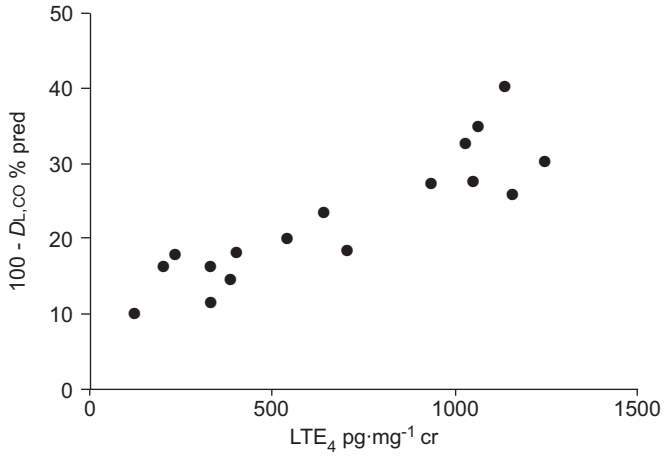

FIGURE 4. Association between concentrations of urinary leukotriene (LT)E_4 and diffusing capacity of the lung for carbon monoxide ($D_{L,CO}$). There was a significant correlation between urinary LTE_4 concentration and 100 - $D_{L,CO}$ (% predicted) in chronic eosinophilic pneumonia patients (r=0.788, p=0.002, n=17).

Interestingly, the $D_{L,CO}$ level is closely correlated with the urinary LTE_4 concentration in CEP patients only during exacerbation of the disease. $D_{L,CO}$ is a useful parameter for detecting and managing diseases affecting the surface area and integrity of the alveolar capillary membrane. In interstitial diseases, the reduced $D_{L,CO}$ is considered to be the earliest abnormal finding, which may be mainly due to the loss of alveolar units rather than to an increase in the thickness of the alveolar capillary membrane [26]. The current study demonstrated that both $D_{L,CO}$ and urinary LTE_4 concentration possibly contribute to the early diagnosis and monitoring of the pathophysiological features of EP. However, in the present study, the authors could not show an apparent relationship between urinary LTE_4 concentration and the severity of the disease.

In conclusion, it has been demonstrated that the progression of eosinophilic pneumonia is associated with elevated urinary leukotriene E_4 and eosinophil-derived neurotoxin concentrations, which may originate from eosinophil activation. The leukotriene E_4 concentration is correlated with the level of diffusing capacity of the lung for carbon monoxide during acute exacerbation. These findings suggest that monitoring of the leukotriene E_4 concentration may aid in the management of eosinophilic pneumonia patients; however, further large-scale studies and intervention studies are necessary to clarify the role of cysteinyl leukotrienes in the pathogenisis of eosinophilic pneumonia.

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