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# Increased urinary leukotriene E4 concentration in patients with eosinophilic pneumonia

Short title: Leukotriene and eosinophilic pneumonia

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## Abstract

**Objective:** Although eosinophils produce cysteinyl leukotrienes (CysLTs) in large quantity, information on relationship between CysLTs and eosinophilic pneumonia (EP) is lacking. We quantified inflammatory mediator concentrations in urine to clarify the relationship between CysLT concentrations and EP severity.

**Methods:** Leukotriene E4 (LTE4), eosinophil-derived neurotoxin (EDN), 9α, 11β-prostaglandin F2 (9α, 11β-PGF2), and LTB4 glucuronide (LTBG) concentrations were quantified in the urine of EP patients during acute exacerbation and clinical remission, asthmatic patients during acute exacerbation and under stable condition, and healthy control subjects.

**Results:** The urinary LTE4 and EDN concentrations of EP patients during acute exacerbation were significantly higher than those of asthmatic patients and healthy subjects, and which immediately decreased during clinical remission. The urinary LTE4 concentration was associated with the urinary EDN concentration of EP patients during acute exacerbation. The urinary LTE4 concentration significantly correlated with the diffusing capacity of the lung for carbon monoxide ($D_{LCO}$) in EP patients during acute exacerbation.

**Conclusions:** The increased urinary concentrations of LTE4 and EDN were associated with acute exacerbation in EP patients. The increased LTE4 concentration significantly correlated with $D_{LCO}$, suggesting that the monitoring of LTE4 concentration may aid in the management of EP patients.

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**Key words:**

biomarkers

bronchial asthma (asthma)

diffusing capacity

eosinophilic pneumonia

eiocosanoids (prostaglandin and leukotrienes)

leukotriene

**Abbreviations:**

EP: eosinophilic pneumonia

AEP: acute eosinophilic pneumonia

CEP: chronic eosinophilic pneumonia

CysLTs: cysteinyl leukotrienes

LTE4: leukotriene E4

9α, 11β-PGF2: 9α, 11β-prostaglandin F2

EDN: eosinophil-derived neurotoxin

LTBG: leukotriene B4 glucuronide

Cr: creatinine

$D_{LCO}$: diffusing capacity of the lung for carbon monoxide

BA: bronchial asthma

HC: healthy control

## Introduction

Eosinophilic pneumonia (EP) is a diffuse infiltrative lung disease characterized 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 E2 (PGE2), IL-5, RANTES, eotaxin, and MCP-1, which are 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 LTD4, LTB4, 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 CysLT concentration (9, 10). Although eosinophils have the capacity to generate LTC4 in large quantity, there are as yet 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 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 LTE4 concentration is considered a good marker of LTC4 production in the human body. Similarly, LTB4 glucuronides hava been used as a marker for the whole-body production of LTB4 (11). Prostaglandin D2 (PGD2) has been identified as the main metabolite of arachidonic acid metabolism by the cyclooxygenase pathway in mast cells. After generation in the body, PGD2 is degraded to 9α, 11β-prostaglandin F2̲ (9α, 11β-PGF2), which is subsequently excreted into the urine. Urinary 9α, 11β-PGF2 is presumably related to mast cell activation (12). EDN is used as an eosinophil degranulation marker in urine. EDN is released from eosinophil granules together with eosinophil cationic proteins and EPO. The molecular weight of EDN is 18-19 kDa, which denotes that EDN is excreted in the urine more easily than EPO, which has a molecular weight of 66 kDa.

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

## Methods

### Subjects

This hospital-based study was conducted from January 2004 through December 2006. The diagnosis of EP was established on the basis of currently used criteria and the patients were classified into 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 in this study: 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 eosinophilic pneumonia (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 the idiopathic hypereosinophilic syndrome. We also excluded the patients who had taken medications such as leukotriene receptor antagonists and oral corticosteroids prior to this study, or patients who had exacerbated asthma for at least 3 months preceding the study. For comparative analysis of urinary mediator data, we enrolled 18 patients with bronchial asthma during acute exacerbation (BA-exacerbation group), 15 patients with bronchial asthma under clinically stable condition (BA-stable group), and 15 healthy control subjects (HC group). The diagnosis of asthma was based on the American Thoracic Society criteria (13). Asthma exacerbation was defined as not only episodes of shortness of breath, cough, wheezing, respiratory distress or some combination of these

symptoms as but also a decrease in forced expiratory volume in one second ($FEV_1$) of 20% or greater from the previous best values by measuring lung function (14). The HC group was enrolled without any subjective symptoms or objective findings of the diseases, including allergic diseases. We confirmed that all the subjects enrolled in this study could tolerate nonsteroidal anti-inflammatory drugs (NSAIDs) on the basis of negative past history and/or aspirin provocation results. Permission to conduct this study was obtained from the Ethics Committee of the Sagamihara National Hospital and all the subjects gave their informed consent.

**Study design**

We collected urine samples between 8:00 and 10:00 AM from EP patients, BA-stable group, and HC group. In particular, during the acute exacerbation of EP or acute asthmatic exacerbation, we collected urine samples before intensive corticosteroid therapy. To confirm the relationship between the clinical conditions and urinary LTE4 concentrations in EP patients, we compared the urinary LTE4 concentrations before and after the therapy. In EP patients, the diffusing capacity of the lung for carbon monoxide ($D_{LCO}$) was measured based on the American Thoracic Society guidelines (15).

**Measurements**

Urine was collected in polypropylene bottles containing 4-hydroxy-TEMPO and the aliquots were stored at -35 □ until analysis. LTE4 was quantified using a commercial enzyme immunoassay (EIA) kit (Cayman, Ann Arbor, MI, USA)

after purification by high-performance liquid chromatography (HPLC) as reported previously (16). EDN was quantified using an EIA kit (MBL, Nagoya, Japan) after diluting the urine 50 times in phosphate buffer (17). 9α, 11β-PGF2 was quantified by EIA (Cayman) after extraction with an Empore C18 disk cartridge, according to the procedure reported by O'Sullivan et al. (18). The LTBG concentration was determined from the LTB4 concentration after hydrolysis with β-glucuronidase as reported previously (19). The LTB4 concentration was determined by EIA (Cayman). The concentrations of all the mediators were normalized to urinary creatinine (cr) concentration.

**Statistical analysis**

Data of the four groups (EP during acute exacerbation, BA-exacerbation, BA-stable, and HC groups) were initially analyzed 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. Differences in the urinary biomarker concentrations of EP patients between exacerbation and clinical remission were evaluated using Wilcoxon *t* test. Relationships were analyzed by the Spearman' rank correlation test. *P* values less than 0.05 were regarded as statistically significant.

## Results

Among the twenty-five subjects who were approached to identify the sample population, we excluded six EP patients among which three had already taken corticosteroids prior the study, two had exacerbated asthma within one month preceding the study and one had suspected Churg-Strauss syndrome. Nineteen idiopathic EP patients, consisting of 2 patients with acute EP and 17 patients with chronic EP, were enrolled in this study. The clinical characteristics of the EP patients are shown in Table 1. Among the seventeen CEP patients, eight had CEP accompanied by bronchial asthma 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 the therapies, the clinical conditions of all the patients improved immediately.

### Urinary LTE4 concentration: comparison among EP patients, asthmatics, and healthy subjects

Figure 1 shows the urinary LTE4 concentrations in all the groups. The urinary LTE4 concentration was significantly higher in the EP patients during clinical exacerbation (including both AEP and CEP patients), (median, 719 pg/mg-cr) than in the BA-exacerbation group (138 pg/mg-cr; $p<0.001$), BA-stable group (79 pg/mg-cr; $p<0.001$), and HC group (66 pg/mg-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, 1827 ng/mg-cr) than in the BA-exacerbation group (886 ng/mg-cr; p=0.038), BA-stable group (522 ng/mg-cr; p=0.022), and HC group (408 ng/mg-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, BA-exacerbation group, BA-stable group, and HC group. Because the patients who participated in this study had normal renal, biliary or metabolic clearance, we compared the concentrations of urinary metabolites after correction for creatinine concentration. The concentrations of LTBG and 9α, 11β-PGF2 were slightly lower than that of LTE4. The concentrations of these metabolites were above the limit of detection of each EIA kit; the manufacturer reported that the limits of detection for LTB4 and 9α, 11β-PGF2 EIAs are 6 and 15 pg/ml, respectively. The concentrations of LTBG and 9α, 11β-PGF2 were 10.2 [7.5-15.6] and 52 [27-382] pg/ml, respectively, without correction using the creatinine concentration.

**Changes in urinary LTE4 and EDN concentrations during the clinical disease course in CEP patients**

We examined the relationship between the concentrations of urinary mediators and the clinical disease course in CEP patients (both CEP-BA group and CEP-alone group) (n=17). As shown in Figure 2, the concentration of urinary LTE4 in CEP patients significantly decreased from 823 [115-8553] to 117

[19-530] pg/mg-cr (median [range], p=0.003) and that of EDN, from 1827 [70-7890] to 840 [20-51999] ng/mg-cr (p=0.021). Data are expressed as the concentrations in clinical exacerbation vs. 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. We found no significant difference in the concentrations of all the mediators measured in this study between the asthmatics (CEP-BA group) and nonasthmatics (CEP-alone group).

**Correlation between urinary LTE4 concentration and EDN concentration in CEP patients**

Next, we examined the correlation between the concentrations of these urinary mediators in CEP patients. As shown in Figure 3, there was a significant correlation between urinary LTE4 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 LTE4 concentration and 9α, 11β-PGF2 concentration, between urinary LTE4 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**

We found a significant correlation between the urinary LTE4 concentration and 100-$D_{LCO}$% (% predicted) in pulmonary functional test during clinical

exacerbation of the disease in CEP patients ($r=0.788$, $p=0.002$, $n=17$) (Figure 4). There was no significant correlation between the concentrations of any other urinary mediators measured in this study and clinical characteristics of CEP patients. In addition, we found no correlation between the urinary LTE4 concentration and the number of eosinophils in peripheral blood or BALF. We found no significant difference in clinical characteristics between the CEP-BA group and CEP-alone group.

## Discussion

In this study, we demonstrated for the first time that the urinary LTE4 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 LTE4 concentration is currently considered as one of the best available markers of in vivo CysLT production (20). The urinary LTE4 concentrations 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 this study, despite their asthma complications, experienced an asthmatic exacerbation or showed any symptoms of airway narrowing. We do not have a satisfactory explanation as to why the EP patients did not show impaired central bronchi function even of 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, LTC4 and PGD2 in asthmatic patients (25). On the other hand, there has been no sufficient evidence that mast cells infiltrate the bronchial smooth muscle of EP patients and 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 LTC4 is produced eosinophils in the lungs of EP patients although the physiological stimuli that trigger eosinophils to generate LTC4 have not been fully elucidated.

Interestingly, the $D_{LCO}$ level is closely correlated with urinary LTE4 concentration in CEP patients only during the exacerbation of the disease. $D_{LCO}$ 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 $D_{LCO}$ 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). This study demonstrated that both $D_{LCO}$ and urinary LTE4 concentration possibly contribute to the early diagnosis and monitoring of the pathophysiological features of EP. However, in this study, we could not show an apparent relationship between urinary LTE4 concentration and the severity of the disease.

In conclusion, we have demonstrated that the EP progression is associated with elevated urinary LTE4 and EDN concentrations, which may originate from eosinophil activation, and the LTE4 concentration correlated with $D_{LCO}$ level during acute exacerbation. These findings suggest that the monitoring of the LTE4 concentration may aid in the management of EP patients although further large-scale studies and intervention studies are necessary to clarify the roles of CysLTs in the EP pathogenesis.

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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 | 2/3/5 | 0/2/6 |
| White blood cells,serum, $\times 10^6$/l | 7220 (4500-9500) | 12580 * (8200-20100) | 11620 * (6000-15300) |
| Blood eosinophils, % | 14 (12-16) | 26 (11-71) | 30 (12-54) |
| CRP, mg/ml | 8.6 | 6.2 | 5.5 |
| Total serum IgE, IU/ml | 31 (15-47) | 172 * (24-1240) | 312 * (63-12500) |
| BAL total cells, $\times 10^5$/ml | 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) |
| $PaO_2$, Torr | 86 (82-89) | 78 (72-95) | 77 (65-85) |
| $FEV_1$, % predicted | 89 (85-92) | 86 (78-117) | 78 (73-92) |
| $D_{LCO}$, % predicted | 81 (75-86) | 82 (65-92) | 76 (55-88) |

Abbreviations: AEP = acute eosinophilic pneumonia; CEP-alone = chronic eosinophilic pneumonia without asthma; CEP-BA = chronic eosinophilic pneumonia with asthma; CRP = C-reactive protein, IgE = immunoglobulin E, BALF = bronchoalveolar lavage fluid; $PaO_2$ = arterial oxygen tension; FEV1 = forced expiratory volume in one second; $D_{LCO}$ = Diffusing capacity of the lung for carbon monoxide.

Data are presented as median (range).

*$p < 0.05$, compared with patients with AEP

TABLE 2 Concentrations of urinary mediators

| | AEP (n=2) | CEP-alone (n=9) | CEP-BA (n=8) | BA-exacer-bation (n=18) | BA-stable (n=15) | HC (n=15) |
|---|---|---|---|---|---|---|
| LTE4 (pg/mg-cr) | 2502*†§ (1952-3051) | 722*†§ (160-5520) | 855*†§ (115-8553) | 138**‡ (80-566) | 79 (50-221) | 66 (34-178) |
| EDN (ng/mg-cr) | 1150**‡¶ (935-1365) | 1850**‡¶ (556-7890) | 2312**‡¶ (70-7582) | 886**‡ (135-1522) | 522 (152-885) | 408 (180-620) |
| 9α, 11β-PGF2 (pg/mg-cr) | 108 (76-140) | 85 (35-136) | 72 (3-1067) | 92 (25-520) | 95 (5-202) | 82 (7-195) |
| LTBG (pg/mg-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) |

Abbreviations: LTE4 = leukotriene E4, EDN = eosinophil-derived neurotoxin, 9α, 11β-PGF2 = 9α, 11β-prostaglandin F2, LTBG = leukotriene B4 glucuronide, Cr = creatinine.

Results are expressed as in Fig 1.

Data are presented as median (range).

* $p<0.001$, ** $p<0.05$, compared with HC group

† $p<0.001$, ‡ $p<0.05$, compared with BA-stable group

§ $p<0.001$, ¶ $p<0.05$, compared with BA-exacerbation group

## Figure Legends

**Figure 1 Urinary LTE4 concentration: comparison among EP patients, asthmatics, and healthy subjects.**

EP: eosinophilic pneumonia, BA: bronchial asthma, HC: healthy controls. EP patients during clinical exacerbation, BA-exacerbation group, BA-stable group, and HC group are designated by closed circles, open triangles, open squares, and open circles, respectively. Horizontal bars indicate medians.

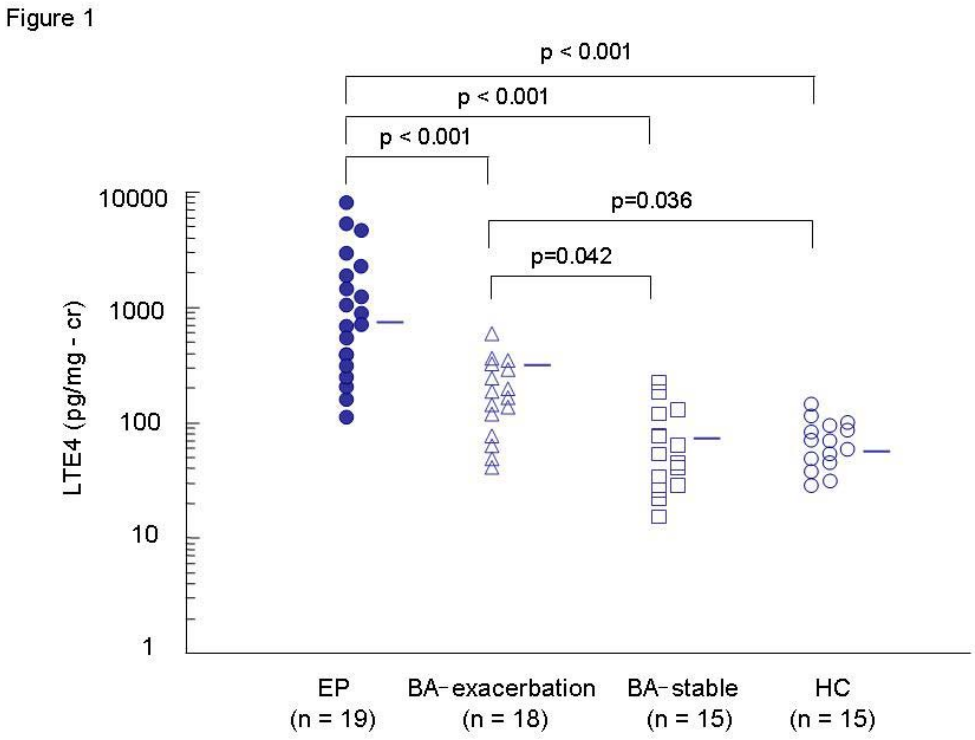



**Figure 2 Changes in urinary LTE4 (A) and EDN (B) concentrations during the clinical disease course in CEP patients.**

CEP-alone (n=9) and CEP-BA (n=8) patients are designated by open circles with solid lines, closed circles with dashed lines, respectively. Horizontal bars indicate medians.

Figure 2

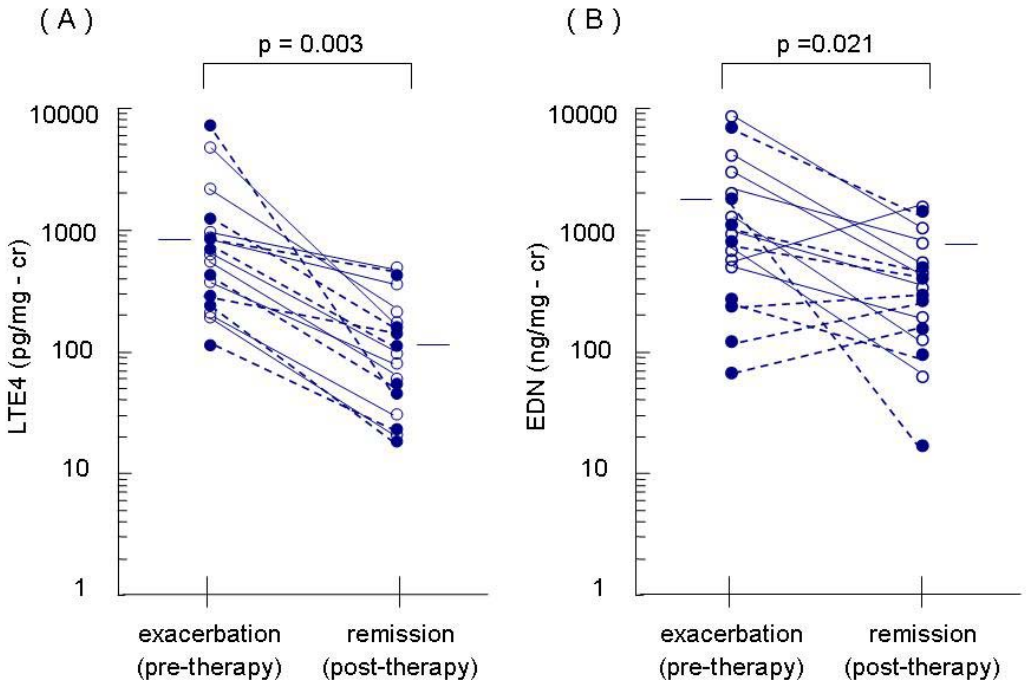



**Figure 3 Correlation between urinary LTE4 and EDN concentrations in CEP patients.**

There was a significant correlation between urinary LTE4 and EDN concentrations during clinical exacerbation (r=0.668, p=0.033, n=17), but not during clinical remission after therapy in CEP patients. CEP patients during exacerbation and clinical remission are designated by closed and open circles, respectively.

Figure 3

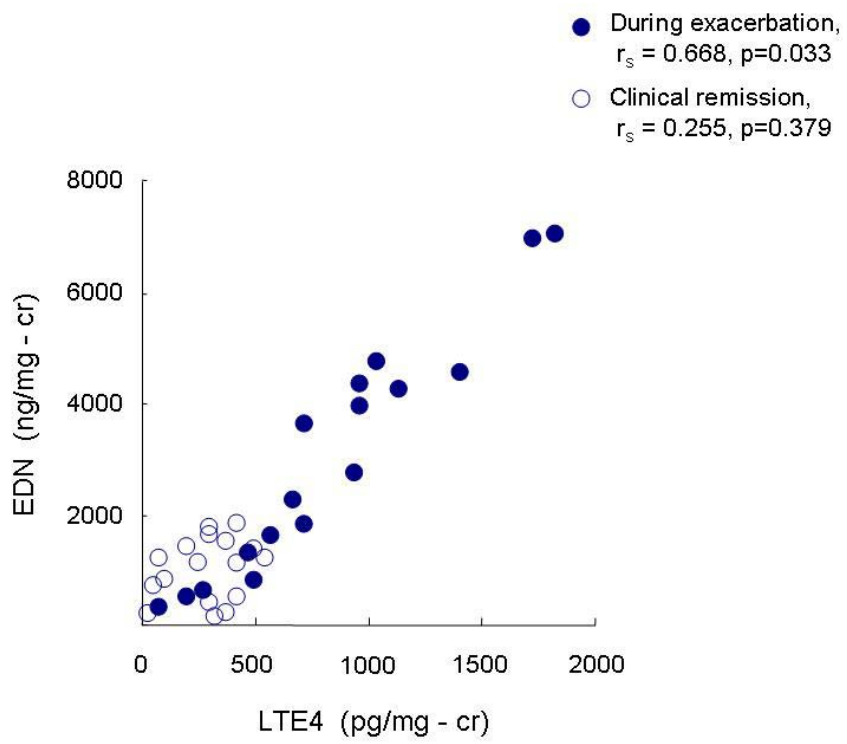
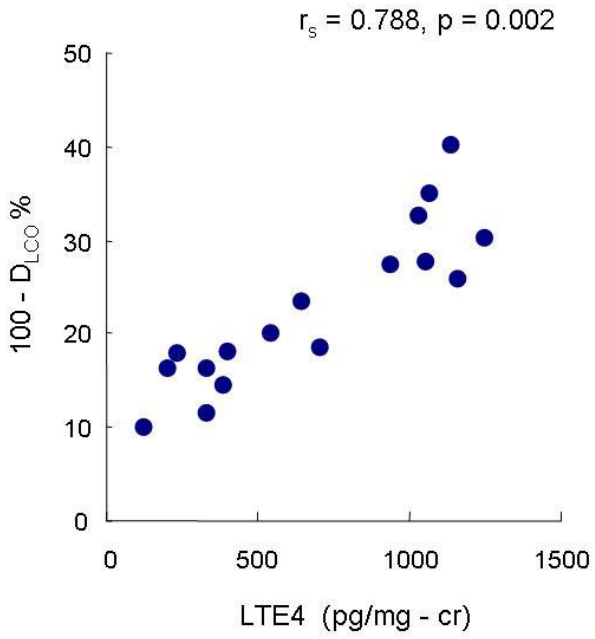



**Figure 4 Association between concentrations of urinary mediators and clinical features of CEP patients.**

There was a significant correlation between urinary LTE4 concentration and 100-$D_{LCO}$% (% predicted) in pulmonary functional test during clinical exacerbation of the disease in CEP patients (r=0.788, p=0.002, n=17).

Figure 4


$r_s$ = 0.788, p = 0.002
50
40
30
20
10
0
100 - $D_{LCO}$ %
0
500
1000
1500
LTE4 (pg/mg - cr)