

Letter to the Editor

Elevated serum osteopontin level is associated with blood eosinophilia and asthma comorbidity in patients with allergic rhinitis*To the Editor:*

Allergic rhinitis (AR) and asthma often coexist and are referred to as “united airways” disease; thus, a bidirectional unified airway model of respiratory diseases has been proposed. Asthma is a chronic inflammatory disease of the airways characterized by eosinophilic airway inflammation, enhanced T_H2 immune response, and airway remodeling. It has been well accepted that AR has an important relationship with asthma susceptibility by worsening the lower airway function in some patients. Numerous progresses in understanding the biology of airway disease have identified inflammation as the key trigger factor for concomitant asthma.¹ However, the molecular and cellular pathways that are crucially involved in the interaction between upper and lower airways remain to be identified.

Osteopontin (OPN) is an extracellular matrix protein with a wide range of functions on tissue fibrosis, tumor progression, as well as immune regulation. OPN is upregulated in a wide variety of human inflammatory diseases, such as idiopathic pulmonary fibrosis, autoimmune uveitis, multiple sclerosis, Crohn's disease, and allergic diseases.² Emerging evidence supports an active role for OPN in T_H2 -linked inflammation in the pathogenesis of asthma. For example, increased levels of OPN have been found in the bronchial epithelial cells and macrophages of patients with asthma.³ OPN is also expressed and functional in the peripheral blood eosinophil (EOS) of atopic human subjects and is involved in the *in vitro* migration of EOS isolated from asthmatic patients.⁴ Moreover, increased levels of OPN have been demonstrated in the nasal mucosa of patients with AR in our previous study.⁵ Therefore, it is of great interest to investigate whether increased OPN level in patients with AR is related to asthma comorbidity in the context of a unified airway.

We enrolled 58 patients with AR and 24 normal controls to address this issue. The clinical characteristics are listed in Table E1 in this article's Online Repository at www.jacionline.org. The study protocols were approved by ethics committee boards, and all subjects gave written informed consent. The venous blood from patients with AR was collected, and serum OPN and IL-5 levels were measured by using an ELISA kit (R&D Systems, Minneapolis, Minn) (see the Methods section in this article's Online Repository at www.jacionline.org for further details). The eosinophilic cationic protein (ECP) level, as an indicator of EOS activation, was examined by using the Unicap system (Phadia, Uppsala, Sweden). The detection limit for OPN, IL-5, and ECP was 312.5 pg/mL, 3.9 pg/mL, and 2.0 ng/mL, respectively. Moreover, EOS number in venous blood was measured and counted with a standard automated cell counter. All data are expressed as the medians and interquartile ranges and analyzed by using the Kruskal-Wallis *H* test and the nonparametric Mann-Whitney *U* test except additional note. Correlations between the various parameters were assessed by the Spearman rank correlation analysis. A *P* value of less than .05 was defined as statistically significant.

Our results showed that serum OPN level, as well as ECP and IL-5 levels and blood EOS number, was significantly increased in patients with AR than in normal controls ($P < .05$) (see Table E1), and the elevated serum OPN level was significantly correlated with total nasal symptom score and visual analog scale score in patients with AR ($P < .05$) (Fig 1, A; see Fig E1 in this article's Online Repository at www.jacionline.org). When we subdivided the patients with AR into 3 groups on the basis of disease duration, we found that there were significant differences in OPN levels among the patients ($P < .05$) (see Fig E2 in this article's Online Repository at www.jacionline.org). These findings therefore provided the evidence that serum OPN level is associated with the clinical severity of patients with AR. Furthermore, we found that serum OPN concentration was significantly correlated with blood EOS number and serum ECP and IL-5 levels in patients with AR ($P < .05$) (Fig 1, B, C, and D), indicating a systemic progress initiated by excessive OPN production. To investigate whether the elevated OPN level was associated with asthma comorbidity and possible mechanisms, we subdivided the patients with AR into 2 groups on the basis of with or without concomitant asthma (asthma⁺ group: *n* = 20; asthma⁻ group: *n* = 38). Consequently, we found that the OPN level in the asthma⁺ group was significantly higher than in the asthma⁻ group ($P < .05$) (Fig 1, E). In agreement with OPN production, blood EOS number and serum ECP and IL-5 levels in the asthma⁺ group were significantly higher than those in the asthma⁻ group ($P < .05$) (Fig 1, E, F, and G). Therefore, our finding provided the clue that elevated serum OPN level in patients with AR might contribute to asthma comorbidity through promoting systemic response initiated by EOS migration and activation.

To further test our hypothesis, we examined the effects of recombinant human OPN on isolated EOS from patients with AR in a transwell system *in vitro* (see the Methods section in this article's Online Repository for further details). We found that recombinant human OPN significantly enhanced EOS chemotaxis, as well as activated EOS as indicated by an increase in the ECP level, in a time- (data not shown) and dose-dependent manner (see Figs E3 and E4 in this article's Online Repository at www.jacionline.org). These effects were significantly inhibited by anti-OPN mAb and LY294002, a phosphatidylinositol 3-kinase-specific inhibitor, suggesting that OPN is able to specifically attract and activate EOS through the phosphatidylinositol 3-kinase signaling pathway. Our findings thus demonstrated a critical role of OPN in driving blood eosinophilia, leading to progression of allergic nasal inflammation toward lower airway and subsequent asthma comorbidity.

The initial mechanism behind the relationship between AR and concomitant asthma has not been well established yet. Our previous study,^{5,6} together with Kleijman et al's report,⁷ has documented that enhanced eosinophilia and increased IL-5 production in nasal mucosa might be risk factors for asthma comorbidity. We hereby provide further evidence that elevated serum OPN level is related to blood eosinophilia and the progress of systemic inflammation, highlighting the possible relevance of excessive OPN production in patients with AR to asthma comorbidity. OPN is known to function both as an extracellular matrix molecule and as a cytokine that contributes to T_H2 -skewed airway diseases. In an asthma mouse model, it has previously been demonstrated that OPN plays

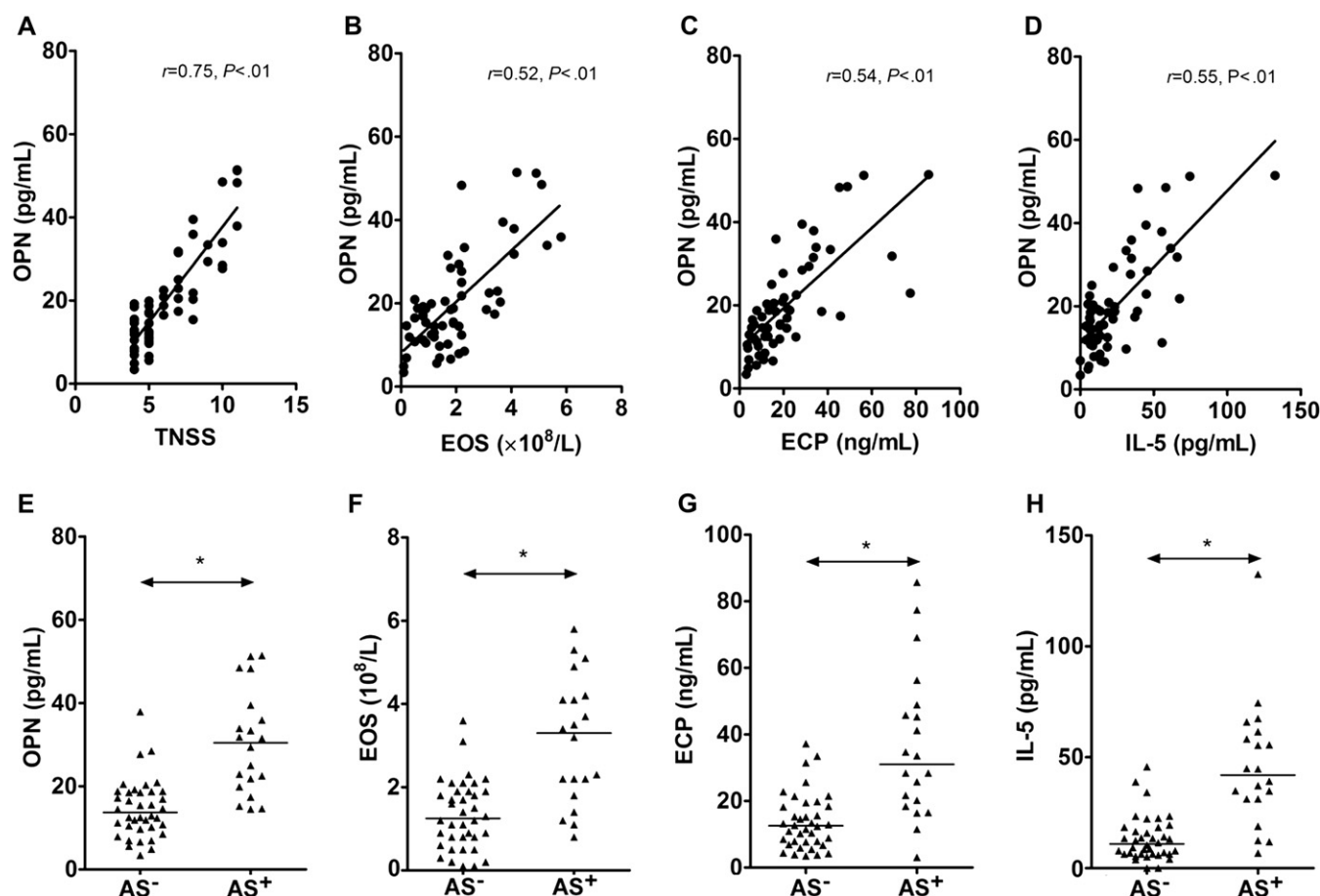


FIG 1. The association between serum OPN concentration and TNSS and other biomarker levels in patients with AR. Serum OPN concentration was positively associated with TNSS (**A**) and blood EOS number (**B**), as well as serum ECP (**C**) and IL-5 levels (**D**) in patients with AR. Moreover, serum OPN concentration (**E**), blood EOS number (**F**), and serum ECP (**G**) and IL-5 (**H**) levels were positively associated with concomitant asthma (AS⁺) in patients with AR. **P* < .05, compared with patients with AR without asthma (AS⁻). AS, Asthma; TNSS, total nasal symptom score.

a crucial role in allergic inflammation by regulating distinct dendritic cell subsets in a time-related manner according to allergen exposure.⁸ In addition, animal studies show that OPN is increased in allergen-induced chronic airway remodeling, whereas its deficiency protects against airway remodeling and bronchial hyperresponsiveness.⁹ However, no studies have investigated the relationship between OPN production and concomitant asthma in patients with AR in a clinical setting. We previously reported that nasal epithelial cells are able to produce excessive OPN through Toll-like receptor-3-mediated signaling pathway after being stimulated with virus mimics.⁴ In this study, we further demonstrated that elevated serum OPN level in patients with AR is associated with EOS migration, activation, and IL-5 overproduction, which might contribute to asthma comorbidity. Taken together, our findings suggested that increased OPN level might be the key proinflammatory factor linking the upper and lower airway by promoting EOS migration and driving the progression of systemic inflammation. Nonetheless, since other indexes of inflammation such as exhaled nitric oxide, urinary leukotriene, and bronchial lavage cellularity were not assessed in the present study, investigation with a larger sample size should be performed to further validate the importance of OPN in patients with AR with concomitant

asthma and to design possible novel strategies for the prevention and treatment of asthma in the unified airway model.

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METHODS

Procedures

Fifty-eight patients with AR who were diagnosed in accordance with Allergic Rhinitis and its Impact on Asthma guideline criteria were recruited for this study. Specifically, AR was defined by the patient's case history, significant nasal symptomatology (sneezing, rhinorrhea, itchy nose, and nasal congestion), and a positive atopic status. The atopic status was evaluated by a skin prick test or the detection of IgE (Phadia) specific to common inhalant allergens (dust mites, pollens, pets, molds, cockroach, etc). Specific IgE values greater than or equal to 0.35 kIU/L were considered positive. The severity of patients with AR was evaluated by total nasal symptom score and visual analog scale score. In brief, individual nasal symptoms including sneezing, rhinorrhea, itchy nose, and nasal congestion were recorded on a 0 to 3 scale (0, no; 1, mild; 2, moderate; and 3, severe) and the total nasal symptom score (0-12) was then calculated. The visual analog scale score was evaluated by a physician on a 0- to 10-cm visual analog scale, with 0 cm being "no complaints whatsoever" and 10 cm being "worst imaginable complaints." Asthma was diagnosed by a pneumologist on the basis of a history of recurrent dyspnea, wheezing, or cough episodes, and either bronchodilator responsiveness, defined as an increase of 12% or greater in FEV₁, increasing 12% or more after inhalation of 400 µg of salbutamol, or airway hyperresponsiveness, defined as a decrease of 20% or greater in the FEV₁ after administration of a cumulative dose of histamine (≤ 7.8 µmol). As normal controls, 24 healthy volunteers were enrolled. All subjects had no oral steroid intake or change of asthma medications 4 weeks prior to study recruitment. All subjects were nonsmokers and free of upper respiratory tract infections for 4 weeks preceding the study. The demographic data from all subjects enrolled in this study are listed in Table E1. This study was approved

by the ethics committee boards, and informed consent was obtained from each subject.

ELISA

Commercially available ELISA kits (R&D Systems, Minneapolis, Minn) were used to measure the serum OPN concentration according to the manufacturer's protocols. The detection limit for OPN and IL-5 was 312.5 pg/mL and 3.9 pg/mL, respectively. For convenient analysis, all values below the detectable limit were considered to be 0.

In vitro EOS chemotaxis and activation assay

Human EOS was isolated from the venous blood of 6 patients with AR by using the magnetic cell sorting-negative immunomagnetic isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions provided by the manufacturer. The purity of EOS was 98% to 100% (Gimesa staining), and the viability was larger than 98% (Trypan blue staining). The isolated EOS was suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Different concentrations of recombinant human OPN (R&D Systems, 0.1-1 µg/mL) were incubated with EOS (10⁵/mL) treated with or without anti-OPN antibody (R&D Systems, 1 µg/mL) in a 24-well transwell system. Recombinant human eotaxin (R&D Systems) was used as positive control of chemotaxis analysis. After incubation, the number of migrated cells was determined by Gimesa staining. Migrated cells were counted in 10 fields by using light microscopy at a magnification of 400×. The ECP level in the supernatant was measured by using the Unicap system (Phadia). LY294002 (10 µM) (Sigma-Aldrich, St Louis, Mo) was used as phosphatidylinositol 3-kinase-specific inhibitor.

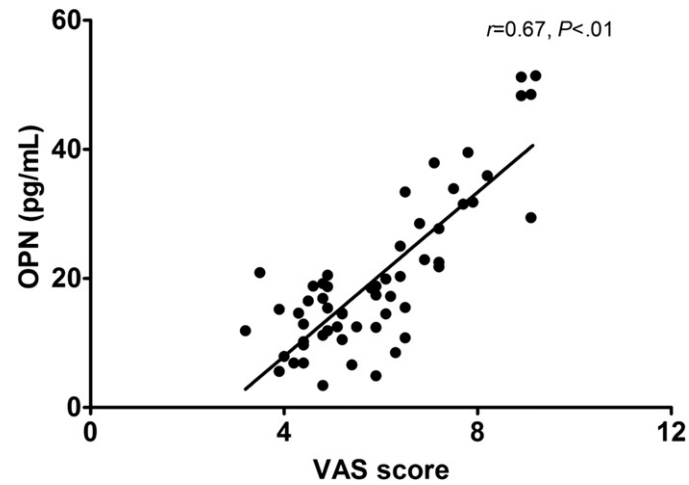


FIG E1. The association between serum OPN concentration and VAS score in patients with AR. Serum OPN concentration was positively associated with the VAS score in patients with AR. * $P < .05$. VAS, Visual analog scale.

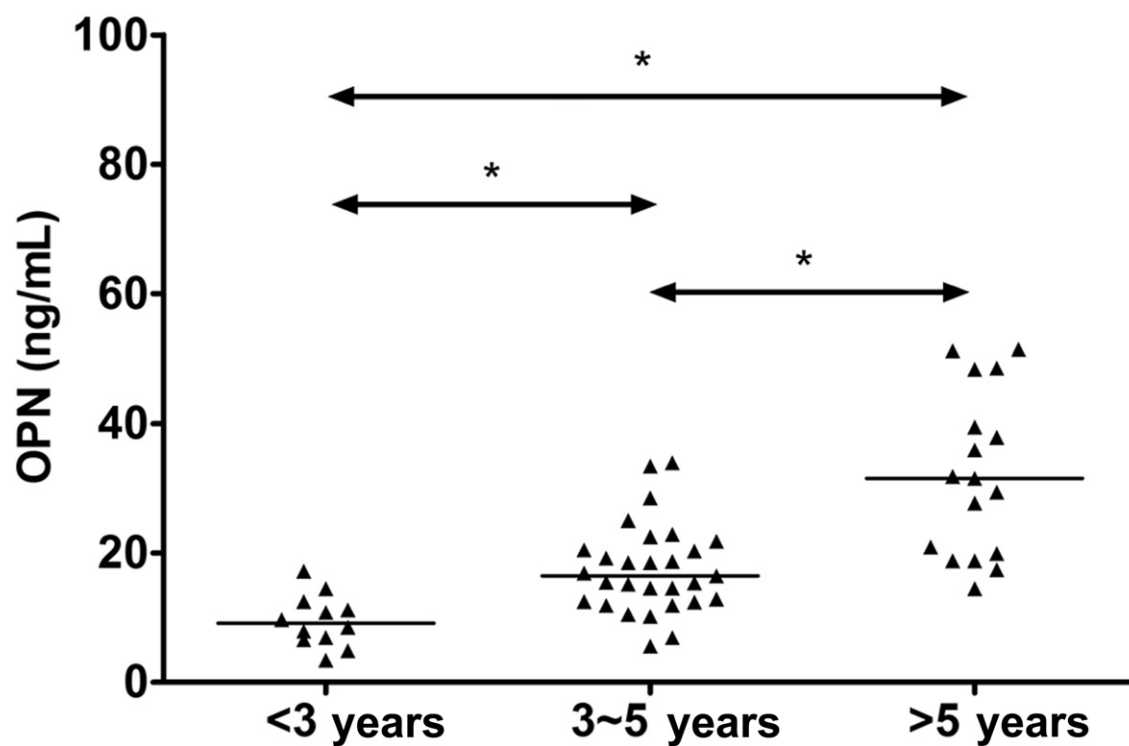


FIG E2. The association between serum OPN concentration and disease duration in patients with AR. Serum OPN concentration was positively associated with disease duration in patients with OR. There were significant differences in OPN levels among 3 groups of patients with AR. The OPN level in group III was significantly higher than in groups II and I (group III, duration >5 years; group II, 5 years $\geq$ duration $\geq$ 3 years; group I, duration <3 years). * $P < .05$.

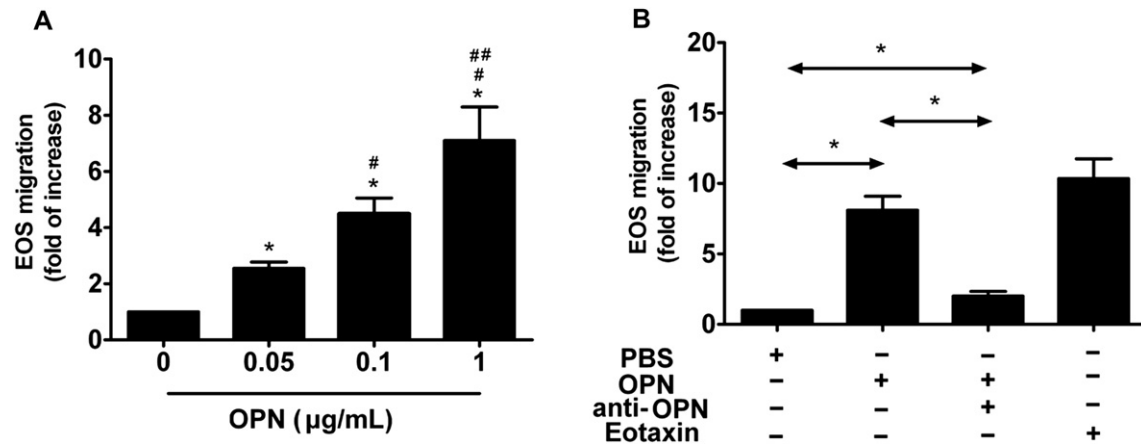


FIG E3. Recombinant human OPN (rh-OPN) enhanced chemotaxis of purified blood EOS *in vitro*. Rh-OPN (0-1 μg/mL) significantly enhanced EOS chemotaxis in a dose-dependent manner after stimulation for 12 hours (**A**); EOS migration has been significantly enhanced by rh-OPN (1 μg/mL), which was similar to the effect induced by 1 μg/mL of recombinant human eotaxin as the positive control; this effect was significantly inhibited by anti-OPN mAb (1 μg/mL) (**B**). The data indicated the means (SEM) of 3 independent experiments. * $P < .05$, compared with the control group; # $P < .05$, compared with the 0.05 μg/mL group; ## $P < .05$, compared with the with the 0.1 μg/mL group.

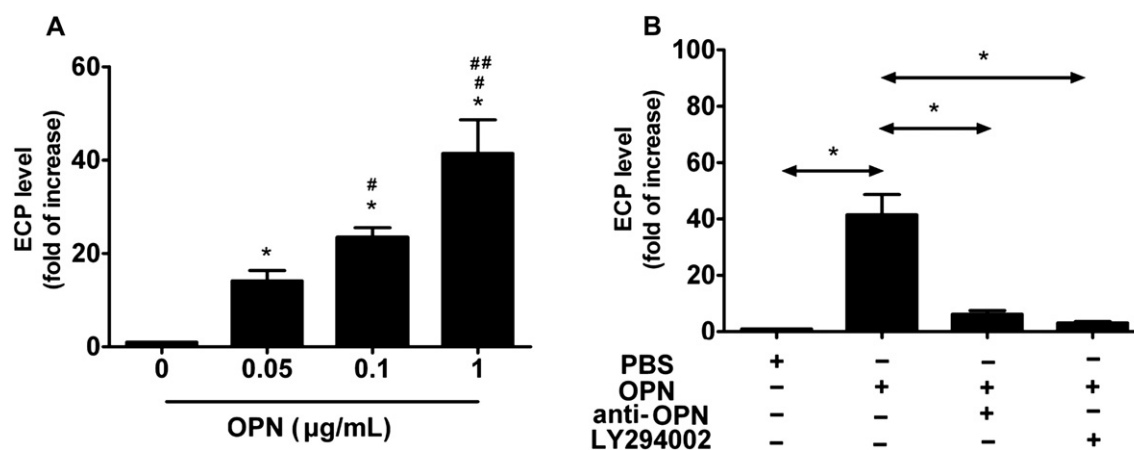


FIG E4. Recombinant human OPN (rh-OPN) enhanced ECP production of purified blood EOS *in vitro*. Rh-OPN (0-1 µg/mL) significantly increased the ECP level released by EOS in a dose-dependent manner after stimulation for 24 hours (**A**). The activated effect of rh-OPN was significantly inhibited by anti-OPN mAb (1 µg/mL) and phosphatidylinositol 3-kinase inhibitor, LY294002 (10 µM) (**B**). The data indicated the means (SEM) of 3 independent experiments. * $P < .05$, compared with the control group; # $P < .05$, compared with the 0.05 µg/mL group; ## $P < .05$, compared with the 0.1 µg/mL group.

TABLE E1. Demographic characteristics of patients with AR and normal controls

Groups	Control	Patients with AR
N	24	58
Sex (M:F)	14:10	32:26
Age (y)	22-30	20-52
Skin prick test-positive	0	58
Asthma in history and present	0	20
Smoking	0	2
Duration of AR (y)	0	1.5-12
TNSS	0	5 (4, 8)*
VAS score	0	5.85 (4.8, 6.95)*
Blood EOS number ($\times 10^8/L$)	0.30 (0.05, 0.45)	1.75 (0.9, 2.3)*
Serum total IgE (kIU/L)	0-13.5	16.4-245.5
Serum specific IgE to D1 (kIU/L)	<0.35	4.1-85.2
Serum specific IgE to D2 (kIU/L)	<0.35	3.2-64.5
Serum ECP level (ng/mL)	0 (0, 6.1)	16.0 (8.8, 28.4)*
Serum OPN level (ng/mL)	1.75 (0.82, 3.65)	17.30 (11.9, 25.68)*
Serum IL-5 level (pg/mL)	4.2 (0, 6.22)	15.05 (7.7, 35.48)*

Age, disease duration, and serum IgE level are shown as range. TNSS, VAS score, blood EOS number, and serum ECP, OPN, and IL-5 levels are expressed as the medians and interquartile ranges.

D1, *Dermatophagoïdes pteronyssinus*; D2, *Dermatophagoïdes farinae*; F, female; M, male; TNSS, total nasal symptom score; VAS, visual analog scale.

* $P < .05$.