

Article Type: Original Article: Airway Diseases

**Title: SPUTUM PERIOSTIN IN PATIENTS WITH DIFFERENT SEVERE ASTHMA PHENOTYPES**

**Short title: Sputum periostin in severe asthma**

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**Keywords:** asthma biomarkers; asthma phenotypes; induced sputum; periostin; severe asthma.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12580

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**Conflicts of interests:** The authors have nothing to declare.

**Declaration of all sources of funding:** This work was partly supported by a competitive grant SEAIC 2011. The research activity of I.B. was supported by Instituto de Salud Carlos III - Fondo de Investigación Sanitaria, programme Río Hortega 2009-2012.

**Contribution of all the authors:**

Irina Bobolea contributed to the conception and design of the study, acquisition of data and analysis and interpretation of data, drafting the article and then revising it critically for important intellectual content and final approval of the version to be published.

Victoria del Pozo and Santiago Quirce contributed to the conception and design of the study, acquisition of data and analysis and interpretation of data, critical revision for important intellectual content and final approval of the version to be published.

Pilar Barranco, Victoria Sanz, David Romero, Valentin López, Carlos Villasante and Julio Canabal contributed to the acquisition of data, critical revision of the article for important intellectual content and final approval of the version to be published.

**TAKE HOME MESSAGE:** Increased sputum periostin levels are associated with fixed airflow limitation in patients with severe eosinophilic asthma.

**Abbreviation list:**

BMI: body mass index

FeNO: Fractional exhaled nitric oxide

FEV<sub>1</sub>: Forced expiratory volume in the first second

MDI: metered dose inhaler

NPAFL: non-persistent airflow limitation

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IgE: Immunoglobulin E

PAFL: persistent airflow limitation

ppb: parts per billion

SPT: Skin prick test

## **Abstract**

### **Background:**

Identifying inflammatory phenotypes is relevant in severe uncontrolled asthma. The aim of the present study was to identify the different clinical, inflammatory, functional and molecular phenotypes in patients with severe asthma and to investigate the potential role of sputum periostin as a biomarker of severe asthma phenotypes.

**Patients and methods:** Sputum induction was performed in 62 patients diagnosed with severe asthma. Skin prick testing, lung function tests, exhaled nitric oxide, hematimetry, and total serum IgE were performed. Periostin was measured in sputum supernatants.

**Results:** Asthma patients were phenotyped and 80% were late-onset asthma, 50% had fixed airflow obstruction, and 66% showed a Th2-high phenotype. With respect to inflammatory phenotypes, 71% were eosinophilic and 25% mixed granulocytic. Periostin levels were higher in patients with fixed as compared to variable airflow limitation (69.76 vs. 43.84 pg/ml,  $p<0.05$ ), and in patients with eosinophilic as compared to mixed granulocytic phenotype (61.58 vs. 37.31 pg/ml,  $p<0.05$ ). There was an inverse correlation between post-bronchodilator FEV<sub>1</sub>/FVC and periostin levels ( $-0.276$ ,  $p<0.05$ ).

**Conclusion:** This study demonstrates the utility of periostin in phenotyping severe asthma. Periostin levels in sputum are associated with persistent airflow limitation in asthma patients with airway eosinophilia despite treatment with high-dose inhaled corticosteroids.

## **Introduction**

It has been estimated that around 10% of asthma patients may be classified as severe (1-3). The current and future approach for asthma management should include stratifying the patients according to the phenotype in order to personalize different asthma treatments (4). In the last decade, remarkable efforts have been made to identify the characteristic features of severe asthma that are different from those described for mild-moderate asthma, setting the stage for the development of new therapies (4). For instance, up to two thirds of the patients with severe asthma have been shown to have persistent sputum eosinophilia in spite of steroid treatment, subtype characterized by late onset, frequent exacerbations, and response to mepolizumab, a monoclonal antibody (mAb) blocking IL-5 (5-7).

Molecular phenotyping of asthma based on Th2 inflammation has important therapeutic implications, as most novel asthma treatments target this pathway. Two new asthma molecular phenotypes, high and low Th2, with significant differences in tissue expression of IL-5 and IL-13, have been described (8). The study of Woodruff et al (8) suggested that the clinical response to anti-IL-13 mAb lebrikizumab may be limited to the specific subgroup of patients with Th2-high asthma, as proven afterwards by Corren et al (9). This Th2-high gene signature includes periostin (encoded by POSTN), a secreted 90-kDa extracellular protein, with structural homology to the adhesion molecule fasciclin I (FAS I), originally detected in osteoblasts (10). Periostin seems to become an emerging biomarker in Th2-driven or eosinophilic inflammation. A study by Jia et al. (11) identified serum periostin as a systemic

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biomarker of airway eosinophilia in severe, uncontrolled asthmatics. Nevertheless, it seems that a more integrative approach, that links clinical features and molecular mechanisms, is the best way to identify asthma phenotypes and its tailored treatments. Bearing in mind this multiscale approach of severe asthma diagnosis, in the present study we measured periostin in the sputum supernatants, as it is far less invasive than bronchial biopsy or bronchoalveolar fluid, but still more organ-targeted than serum periostin, in order to better clarify the role of periostin as an asthma biomarker.

Therefore, the aim of the present study was twofold: first, to identify the different clinical, inflammatory, functional and molecular phenotypes in a group of patients with severe asthma, attended in a specialized Severe Asthma Unit; and second, to investigate the potential role of sputum periostin as a biomarker of the different severe asthma phenotypes.

## **PATIENTS AND METHODS**

### *Study design and patients*

This was a prospective, observational study, performed in a specialized Severe Asthma Unit including specialists in Allergology and Pneumology, in a tertiary care hospital in Madrid, Spain.

Inclusion criteria: Patients  $\geq 18$  years old diagnosed with severe refractory asthma according to ATS criteria (12) were recruited consecutively between October 2010 and June 2012. Moreover, in order to be included in the study, on the day of the sputum induction patients needed to have a post-bronchodilator  $FEV_1 > 50\%$  predicted and  $> 1L$ , for safety reasons, as described elsewhere (13), taking their usual treatment, which was thoroughly recorded and had to have remained unmodified within the last 4 weeks prior to the test.

We excluded smokers >30 pack-year and/ or a chronic obstructive pulmonary disease (COPD) diagnosis, and patients with respiratory infection and/or unstable asthma in the previous 4 weeks.

This study was conducted according to Good Clinical Practice (GCP) standards and the Helsinki Declaration, and it was approved by the Ethics Committee of the Hospital Universitario La Paz. All patients provided written informed consent to their participation in the study, as well as for the sputum induction procedure. All the visits were integrated into the routine practice to assess the evolution of severe asthma patients.

### *Methods*

Demographic and clinical data were collected: age, sex, body mass index (BMI), smoking status; clinical characteristics of asthma (age of onset, asthma exacerbations, symptoms control and asthma-related medication etc.), coexisting conditions and co-morbidities such as aspirin/non-steroidal anti-inflammatory drugs (NSAIDs) hypersensitivity, chronic rhinosinusitis (CRS), nasal polyposis (NP), gastroesophageal reflux, allergic rhinitis and obesity.

We performed skin prick tests, lung function tests, exhaled nitric oxide fraction (FeNO), induced sputum, blood analyses (absolute eosinophil count in peripheral blood, total serum IgE, and all the other diagnostic procedures comprised within the Unit's protocol (i.e. thorax and paranasal sinuses CTs, ENT examination including sinus endoscopy etc), in order to diagnose or exclude co-morbidities.

### *Skin prick tests*

Skin tests were performed on every patient using the prick method (14). Common environmental allergens, including house dust mites, grass, weed and tree pollen, molds (*Alternaria*, *Cladosporium*, and *Aspergillus*), and animal dander (cat and dog) (ALK-Abelló, Madrid, Spain) were tested, as well as histamine (10 mg/ml) and physiologic saline solution (0.9%) as positive and negative controls, respectively. Atopy was defined as the presence of at least one skin prick test to common allergens, with a wheal diameter of 3 mm or greater than the negative control (14).

### *Spirometry*

Spirometry was performed using a Flow Screen spirometer (Vyasis Healthcare GmbH, Hoechberg, Germany) according to American Thoracic Society criteria (15). The spirometer was calibrated daily with a 3-L precision syringe (Vitalograph Ltd, Buckingham, UK). Spirometry parameters Forced expiratory volume in 1 second (FEV<sub>1</sub>), forced vital capacity (FVC), in absolute value and % of predicted, and FEV<sub>1</sub>/FVC ratios were measured and recorded. All patients underwent a bronchodilator test by inhaling 400 mcg salbutamol MDI. Bearing in mind that all patients were receiving high doses of inhaled corticosteroids ( $\geq 1000$  mcg of fluticasone or equivalent) and had to be stable within the 4 weeks prior to the study, baseline and post-bronchodilator FEV<sub>1</sub>/FVC ratios were used to divide the patients into 2 groups (16): those with chronic, persistent airflow limitation (PAFL), also known as fixed obstruction, defined by a FEV<sub>1</sub>/FVC post-bronchodilator  $\leq 70\%$  on two annual visits; and those with normal FEV<sub>1</sub>/FVC ratio  $> 70\%$ , named no PAFL (NPAFL) (17).

### *FeNO measurement*

FeNO was measured by electrochemical reaction using a portable nitric oxide analyzer (NioxMino™, Aerocrine, Solna, Sweden), which provides measurements at an exhalation flow rate of 50 ml/s (18). Baseline FeNO was obtained just before the first spirometric maneuver of the sputum induction procedure.

### *Sputum induction and processing*

Sputum induction was performed using inhalation of hypertonic saline at 4.5% for a maximum period of 20 minutes, with the help of a ultrasonic nebulizer (*Omron NE-U17*), previous administration of salbutamol 400 mcg MDI; the sputum samples were collected and then processed and examined for nonsquamous cell counts by means of cytospin and visual count, following European Respiratory Society recommendations (19), within 2 hours after induction. Periostin was measured in sputum supernatants frozen at -80 °C (*DuoSet ELISA-R&D Systems, Minneapolis, MN*). Prior to the test, sputum supernatants were treated to remove cellular debris and other contaminants that could have interfered with the assay. In all patients, periostin levels were measured in neat supernatants in duplicate. The specificity of periostin assay is 100% and detection limit is 180 pg/ml. We carried out several experiments in order to analyze the possible effect of dithiothreitol (DTT) on periostin measurements, and we observed that this compound used during sputum processing had no effect whatsoever. Thus, we used as diluent reagent, the recommended by the periostin kit ( 1%BSA in PBS, pH 7.2, 0.2 µm filtered), but added DTT in similar concentration as present in the sputum supernatants and compared these results with those obtained without adding DTT and we did not observe any significant differences (data not shown).

The absolute eosinophil count in peripheral blood (cut-off value 140 cells/mm<sup>3</sup>) and the total IgE (cut-off value 100 IU/mL) (CAP KU/L, ThermoFisher, Uppsala, Sweden) were used to divide the patients into the recently-described asthma molecular phenotypes, high and low Th2 (8).

A database was designed to collect data on the demographic and clinical characteristics, treatment patterns and results of all tests performed. The patients' identities remained confidential, each patient being identified solely by a single number assigned by the study coordinator.

#### *Statistical analysis*

The categorical variables were described as absolute and relative frequencies, while continuous variables were reported as the mean and standard deviation or medians and quartiles. Results are expressed as mean  $\pm$  standard deviation, except for FeNO and IgE levels which are expressed as median and interquartile range (IQR) as they did not follow a normal distribution.

Comparison of the quantitative variables was based on the use of non-parametric tests (Wilcoxon test). The Chi-squared test and McNemar test were used to compare the categorical variables. Comparisons between groups were performed with Student's t test for normally distributed variables and with the Mann-Whitney U test for non-normally distributed variables. The Kruskal-Wallis test was used to compare differences between various groups. Data were analyzed with SPSS v11.0 software (SPSS, Inc., Chicago, IL).

## RESULTS

### Clinical characteristics of the subjects

A total of 62 patients diagnosed with severe refractory asthma following the ATS criteria (12), with post-bronchodilator FEV<sub>1</sub>>1L and >50% predicted the day of the sputum induction were initially included in the study. Sputum induction was successful in 52 patients (83%). The rest of them failed to expectorate after 20 minutes of stimulation, or the sample was considered inadequate (squamous cells>15% and/ or cell viability <70%) and those patients were excluded from the study. The demographic, clinical, pulmonary function and inflammatory characteristics of the 52 patients are presented in Table 1. Regarding exacerbations, an important marker of asthma control (future risk), we found that 92% of the patients had had at least one exacerbation in the year prior to their inclusion in the study, but in fact the vast majority (72% of the patients) had had at least 3 exacerbations.

### Phenotyping asthma subjects

In order to classify the asthmatic patients into different phenotypes, we considered the following variables: Late-onset ( $\geq 12$  years) versus early-onset ( $< 12$  years) asthma (5), molecular phenotypes Th2-high and -low (8, 9), persistent versus non persistent (variable) airflow limitation (PAFL vs NPAFL) (16), and obesity-related asthma (body mass index [BMI] $\geq 30$  kg/m<sup>2</sup>).

As for the inflammatory phenotypes, they were defined by differential sputum cells counts according to SARP (20) cut-off values for eosinophils (2%) and neutrophils (40%). Fifty patients had sputum eosinophilia  $> 2\%$ . The results are shown in Table 2.

In order to better characterize the identified asthma phenotypes, we performed a series of comparisons of continuous and categorical variables between the-subgroups according to the clinical and functional phenotypes. Briefly, the statistically significant or otherwise relevant results are summarized below.

#### *Comparisons of variables between subgroups*

Early-onset asthma (20%) versus late-onset (80%): a significantly higher BMI was observed in late onset, and a higher FeNO in early onset asthmatics ( $p<0.05$ ).

Subgroups PAFL vs NPAFL: no significant differences in mean age, Th2 high/low, presence or absence of atopy, age of onset of asthma symptoms, BMI and FeNO levels were found between these subgroups.

No statistically significant differences were found between the two eosinophilic asthma phenotypes (pure eosinophilic and mixed granulocytic) for all the compared variables, except for the periostin levels, as mentioned below.

#### **Sputum periostin as biomarker of asthma phenotypes**

In order to study if sputum periostin might be a biomarker of different asthma phenotypes, we compared sputum periostin levels in each pair of phenotypes. As can be observed in figure 1B, the periostin levels were significantly higher in patients with eosinophilic as compared to those with mixed granulocytic inflammatory phenotype (61.58 [3.1-152] vs 37.31[0.1- 97 ] pg/ml,  $p<0.05$ ).

When we compared the periostin levels between the 25 PALF and 25 NPALF patients with persistent sputum eosinophilia in spite of treatment with high-dose inhaled corticosteroids, we found that periostin levels were significantly higher in patients with fixed as compared to

those with variable airflow limitation (PAFL versus NPAFL, mean [range ]) (69.76 [3.1-152] vs 43.84[0.1-117] pg/ml,  $p<0.05$ ; Figure 1 D), and also that there was an inverse correlation between FEV<sub>1</sub>/FVC post-bronchodilator and periostin levels (-0.276,  $p<0.05$ ).

Nevertheless, periostin levels were similar in non-obese as compared to obese asthmatics, in Th2 high as compared to Th2 low patients and in early-onset compared to late-onset (Figure 1 A, C, E).

A weak positive correlation (0.313,  $p= 0.02$ ), was found between periostin levels and the sputum eosinophilia % across all the study patients, but not between FeNO levels ppb and periostin levels (0.064,  $p= 0.667$ ).

## DISCUSSION

Most of the severe asthma patients in our series (96%) had sputum eosinophilia. Periostin levels in sputum were associated with persistent airflow limitation, but were similar in Th2 high as compared to Th2 low patients, and we could not demonstrate any correlation between sputum periostin and FeNO levels.

The distinction between phenotypes based on inflammatory markers has been improved by the introduction and use of noninvasive techniques for measuring airway inflammation, such as induced sputum, FeNO and exhaled breath condensate, as well as by the identification of some biomarkers in peripheral blood and urine (21, 22). Induced sputum is a reproducible, validated, relatively simple and cheap technique for the assessment of airway inflammation in severe asthma and results particularly interesting as it makes possible to analyze both the cells present in the airways and countless mediators in the supernatant.

The Severe Asthma research Program (SARP) was launched in the USA in an attempt to identify and characterize a large number of patients meeting the ATS severity criteria (1, 2). A subanalysis of the SARP focused on 175 patients with severe asthma (20) sought clinical and pathophysiological differences between patients phenotyped according to the cellularity of induced sputum as a marker of underlying inflammation. It has been shown that both the presence in the sputum of >2% eosinophils, as of > 40% neutrophils, related to poorer lung function, increased morbidity and exacerbations. The "eosinophilic phenotype", as compared to the "neutrophilic phenotype," presented higher FeNO values, more peripheral eosinophilia and increased need for rescue medication. Patients who met both inflammatory features were the most severe. As our study population resembled more the SARP subgroup, we chose these cut-off points for sputum eosinophilia and neutrophilia, respectively, when phenotyping the patients. Thus, by analysis of differential sputum cell count, we found that 96% of the patients with severe asthma had airway eosinophilia ( $\geq 2\%$ ) despite of receiving treatment with high-dose inhaled corticosteroids, percentage higher than in other series (1, 7, 23). This quasi absence of the "pure" neutrophilic phenotype could be explained on one hand by the high percentage of atopy, Th2-high profile and nasal polyposis/hypersensitivity to aspirin in our study population, and, on the other hand, by having thoroughly excluded COPD/heavy smokers and patients with severe lung function impairment, among whom the neutrophilic phenotype could have been expected.

Recently, Wenzel published a paper (24) which takes a new approach to the asthma phenotypes, evolving from the clinical approach to the molecular mechanisms. Regardless of all phenotypic studies published, it seems that the age of onset was a factor of utmost importance. The early onset consistently associated with an allergic condition across all ranges of severities. Late onset is usually less allergic and is associated with persistent

eosinophilic inflammation, obesity and female sex, as we also found in our study (80% of our patients were women, >60% obese, 62% non-allergic endotype).

Mounting evidence supports the emergence of periostin as a potentially valuable biomarker for Th2-driven airway inflammation (25). Periostin, which is secreted by the airway epithelial cells in response to stimulation by IL-4 and IL-13, was originally identified by genome-wide profiling in bronchial biopsies (10). Periostin, independently or in combination with other more conventional biomarkers, such as blood and sputum eosinophils, FeNO, and serum IgE, has the potential to better refine current asthma phenotypes. Furthermore, periostin also holds promise in predicting responders to new or existing biological Th2-directed therapies for the treatment of asthma (9). Yet, despite these advances, use of periostin alone or in combination with other biomarkers needs further study to better delineate its utility in characterizing asthmatic patients by phenotype and predicting their response to new or existing treatments.

The study published by Jia et al (11) showed that serum periostin was a good predictor of eosinophilic airway inflammation. Mean periostin levels were significantly higher in ‘‘eosinophil-high’’ as compared to ‘‘eosinophil-low’’ subjects, as defined by sputum or tissue eosinophil measurements. In a logistic regression model, serum periostin was the single best predictor of sputum and tissue eosinophilia, showing superiority to blood eosinophils, IgE, and FeNO.

In our study, however, no correlation was found between FeNO and periostin levels ( $r=0.064$ ,  $p=0.667$ ), but the periostin levels were significantly higher in patients with eosinophilic as compared to those with mixed granulocytic inflammatory phenotype, suggesting that multiple factors may affect and determine sputum periostin concentrations.

On the other hand, as previously suggested by other authors (11,26), periostin seems to be

associated with airway remodeling, especially with subepithelial fibrosis, and with progressive decline in FEV<sub>1</sub>. In our study high sputum periostin levels correlated with fixed airflow limitation. It seems reasonable that differences in airway remodeling may provide an alternative or, more probably, an additional mechanism responsible for the disease activity in the severe asthma population. Pepe et al. (27) for instance consider that smooth muscle alteration is the key structural change that distinguishes severe from moderate asthma, contributing to the difficulty in obtaining adequate control in these subjects.

This study has several limitations, first of them being the small sample size compared to previously published papers (9,20). In exchange, our paper indeed describes the “real world” of patients with severe asthma, managed according to the current asthma management guidelines as opposed to the rigorously controlled conditions of randomized clinical trials. Our results nevertheless are similar with those reported by authors, who also find that increased Th2 airway inflammation is characterized by more severe airflow obstruction (26, 28). Sputum allows a continuous measure of Th2 genes expression that provides information complementary to sputum eosinophil counts (29), nevertheless these tests are expensive, laborious and available only for research purposes in specialized centres, while sputum periostin can be easily and more economically measured by ELISA.

In summary, the percentage of eosinophilic asthma in our patients with severe asthma is higher than in other series, probably due to the strict inclusion and exclusion criteria and to the sample characteristics. To our knowledge, this is the first study that demonstrates the association between sputum periostin levels and persistent airflow limitation in patients with severe asthma and persistent sputum eosinophilia in spite of treatment with high-dose inhaled corticosteroids.

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**Table 1.** Demographic, clinical, lung function and inflammatory features of subjects studied.

|                                                         | Severe asthma subjects<br>n=52 |
|---------------------------------------------------------|--------------------------------|
| Age (mean, range)                                       | 55 (13-68)                     |
| Male                                                    | 20%                            |
| BMI (kg/m <sup>2</sup> ; mean, range)                   | 28 (20-45.3)                   |
| Chronic Rhinitis /Rhinosinusitis (CRS)                  | 67%                            |
| CRS with Nasal polyposis                                | 30%                            |
| Hypersensitivity to aspirin/NSAIDs                      | 20%                            |
| Atopy                                                   | 57%                            |
| Smokers and ex-smokers, both <10 pack-year history      | 44%                            |
| Current smokers. 9 patients                             | 17%                            |
| Ex-smokers, 14 patients                                 | 27%                            |
| GERD                                                    | 86.5%                          |
| Late-onset asthma >12 years                             | 80%                            |
| FEV <sub>1</sub> baseline (% predicted; mean, range)    | 75.8% (34-110)                 |
| FEV <sub>1</sub> /FVC baseline (mean, range)            | 68% (41-89)                    |
| FEV <sub>1</sub> /FVC post-bronchodilator (mean, range) | 68.5% (41-91)                  |
| Current treatment, n (%)                                |                                |
| LABA/IC                                                 | 49 (95%)                       |
| Leukotriene antagonists                                 | 31(59%)                        |
| Oral steroids                                           | 16 (30%)                       |
| Tiotropium                                              | 26(50%)                        |
| Omalizumab                                              | 5 (9%)                         |
| IC dose (mean mcg fluticasone or equivalent)            | 1332                           |
| Oral steroids (mean mg cortisone or equiv.)             | 25                             |
| ACT score, mean ( min-max.)                             | 14 (6-23)                      |
| Exacerbations*                                          | 92%                            |
| FeNO (ppb; mean, range)                                 | 50.21 (4-224)                  |
| Total IgE (IU/mL; mean, range)                          | 288 (3-3698)                   |
| Peripheral blood eosinophil (eos/mm <sup>3</sup> )      | 377 (50-1190)                  |
| Sputum Periostin (pg/ml; mean, range)                   | 53.6 (0.1-152)                 |

**ACT:** Asthma Control Test; **BMI:** body mass index; **FeNO:** Fractional exhaled nitric oxide; **FEV<sub>1</sub>:** Forced expiratory volume in 1 second; **FVC:** forced vital capacity; **GERD:** gastro-esophageal reflux disease; **IC:** inhaled corticosteroids; **IgE:** Immunoglobulin E; **LABA:** long-acting betaagonists; **ppb:** parts per billion.

\* At least one severe exacerbation in the year prior to the inclusion in the study [23].

**Table 2.** Asthma phenotypes.

|                                    | <b>Subjects (%)</b>                              |
|------------------------------------|--------------------------------------------------|
| <b>Obesity-related asthma</b>      | 60                                               |
| <b>Late-onset asthma</b>           | 80                                               |
| <b>PAFL</b>                        | 50                                               |
| <b>Th2 high</b>                    | 66                                               |
| <b>Leukocyte phenotype:</b>        |                                                  |
| <b>-Eosinophilic</b>               | 71                                               |
| <b>-Mixed granulocytic</b>         | 25                                               |
| <b>-Neutrophilic</b>               | 2                                                |
| <b>-Paucigranulocytic</b>          | 2                                                |
| <b>Total cell count/ ml sputum</b> | 1.8 x 10 <sup>6</sup> (0.5-5.8x10 <sup>6</sup> ) |
| <b>% squamous cells</b>            | 11 (4- 15%)                                      |
| <b>% eosinophils</b>               | 22.7 (1.8-42%)                                   |
| <b>% neutrophils</b>               | 29.8 (6-71)                                      |
| <b>% rest (mostly macrophages)</b> | 47.5 (16-64%)                                    |

**PAFL:** persistent airflow limitation.

**Figure 1. Sputum periostin levels in the identified asthma phenotypes.**

Human periostin levels in induced sputum samples were measured by specific ELISA as described in Methods section. Individual data are shown. Statistical analysis was performed by Mann-Whitney *U* test. PAFL: persistent airflow limitation; PHENOTYPE EOS: eosinophilic phenotype; PHENOTYPE MIX: mixed granulocytic phenotype.

