

# RANTES in Exhaled Breath Condensate of Patients with Severe Persistent Allergic Asthma during Omalizumab Therapy

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## Key Words

Omalizumab · Asthma · RANTES · Airway inflammation ·  
Exhaled breath condensate

## Abstract

**Background:** Omalizumab is a humanized monoclonal anti-IgE antibody developed for the treatment of IgE-mediated diseases, including asthma. The aim of the study was to determine the effect of omalizumab treatment on changes in RANTES in exhaled breath condensate and other inflammatory markers in patients with persistent severe asthma.

**Methods:** The study was conducted on a group of 19 patients with severe persistent allergic asthma treated with conventional therapy (according to GINA 2006) and with or without omalizumab (9 vs. 10 patients). Changes in inflammatory parameters [RANTES in exhaled breath condensate, exhaled nitric oxide, blood eosinophil count and serum eosinophil cationic protein (ECP)] were measured before and after 16 weeks of therapy. **Results:** Omalizumab-treated patients showed a statistically significant decrease in the concentrations of RANTES in exhaled breath condensate, exhaled nitric oxide ( $F_{ENO}$ ), serum ECP, and blood eosinophil count compared with patients with conventional therapy after 16 weeks of treatment. In this group of patients, statistically significant correlations were revealed between the de-

crease in RANTES and a decrease in  $F_{ENO}$  and between the decrease in  $F_{ENO}$  and a decrease in ECP or blood eosinophil count after omalizumab therapy. **Conclusions:** Our results confirmed that during anti-immunoglobulin E therapy with omalizumab in patients with severe persistent allergic asthma, RANTES expression is decreased. This process in turn could lead to a limitation of airway inflammation and could be essential for the beneficial effect of anti-IgE therapy with omalizumab.

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## Introduction

Asthma is a chronic inflammatory disease of the airways characterized by infiltration of the mucosa by inflammatory cells, mucus hypersecretion, fibrosis, smooth muscle hypertrophy, epithelial loss, and angiogenesis [1]. These processes lead to variable airflow obstruction responsive to inhaled corticosteroids and  $\beta_2$ -agonists. However, some patients with severe disease, despite conventional treatment, still have poorly controlled disease.

Omalizumab is a monoclonal anti-immunoglobulin E antibody developed for the treatment of patients with inadequately controlled severe persistent allergic asthma despite optimal controller therapy [2]. By reducing serum

IgE levels and expression of FcεRI receptor on key cells in the inflammatory cascade, omalizumab blocks the release of inflammatory mediators from mast cells and reduces the infiltration of inflammatory cells, especially eosinophils, into the airways [3].

Eosinophilic infiltration into affected tissue is one of the hallmarks of allergic inflammation and exacerbation of asthma [4]. Coordination of cytokines such as interleukin 5 (IL-5), eotaxin, and RANTES seems to be necessary for eosinophil extravasation including adhesion, chemotaxis, and activation [5]. In vitro studies have demonstrated the specificity of these eosinophil-selective cytokines for the attraction and activation of eosinophils, and imply their participation in the specific recruitment of eosinophils to sites of allergic inflammation [6].

The mechanisms underlying the clinical and anti-inflammatory efficacy of omalizumab are not fully understood. There are no data assessing the effect of anti-immunoglobulin E treatment on the expression of chemokines, including RANTES, playing an essential role in the development of allergic, particularly eosinophilic, inflammation in asthmatic patients.

The aim of the study was to assess changes in RANTES concentrations in exhaled breath condensate (EBC) of asthmatics with severe persistent allergic asthma during omalizumab therapy and to establish the possible correlation of these measurements with other parameters of airway inflammation.

## Material and Methods

### *Patients*

The study was conducted on a group of 19 patients with severe persistent allergic asthma.

All patients used inhaled short-acting  $\beta_2$ -agonists (as rescue medication), inhaled long-acting  $\beta_2$ -agonists, high doses of inhaled steroids and leukotriene receptor antagonists. Patients did not take oral steroids, at least not in the preceding 2 months and during the study. There were no changes in the patients' treatment during the study. It was an open-label study. Randomization was not performed. Nine patients with severe persistent allergic asthma received omalizumab therapy. Subsequently, 10 patients with severe allergic persistent asthma were included in the group treated with conventional treatment recommended by GINA 2006 [7] without omalizumab. The authors attempted to make these two groups comparable regarding the severity and type of asthma, spirometric indices, intensity of day and night symptoms, controlling treatment used, as well as the need for rescue medication and other parameters used in asthma control assessment. All the patients were atopic and sensitized to common inhaled allergens as evaluated by skin prick tests; they had total serum IgE of at least 30 to no more than 700 IU/ml. All patients had been in a stable condition, free from acute exacerbations and respiratory tract in-

fections for the prior 2 months. They were nonsmokers and during the preceding year had not been passive smokers.

All patients were examined by a physician; then they underwent EBC collection, exhaled nitric oxide ( $F_{ENO}$ ) measurement, and spirometry. Blood samples were collected to determine serum total IgE, eosinophil cationic protein (ECP) and blood eosinophil count. All measurements were performed before and after a 16-week treatment period.

Patients were treated with omalizumab on the basis of the concentration of serum total IgE and patient body weight at baseline [8]. The study protocol was approved by the Ethics of Research Committee of the Medical University of Białystok, agreement No. R-I-002/68/2007. Informed consent was obtained from every patient who entered the study.

### *Measurements*

$F_{ENO}$  was measured in all asthma patients and healthy subjects by the chemiluminescence technique using a Sievers 280i NO Analyzer (Boulder, Colo., USA). The measurements were performed at an expiratory flow of 50 ml/s [9]. The duration of exhalation had to be at least 6 s to produce a stable NO level for 3 s. All subjects had three recorded  $F_{ENO}$  measurements. Repeated measurements were performed until the 3 values agreed within 10% of the mean. The mean value of the three measurements was recorded as the final  $F_{ENO}$  level.

Baseline spirometry was performed using a MasterScreen Pneumo PC spirometer (Erich Jaeger GmbH, Höchberg, Germany). It was performed according to ATS standards [10]. Forced expiratory volume in 1 s ( $FEV_1$ ) was evaluated. Before the examination the patients did not take any medications that could change spirometry results. The highest value of three technically satisfactory attempts was recorded.

EBC was collected by using a commercially available condenser (EcoScreen; Erich Jaeger) according to current ATS/ERS guidelines [11]. All measurements were performed at the same time (between 8.00 and 10.00 a.m.) to avoid a possible circadian rhythm of mediator concentrations in EBC. All patients were asked to refrain from eating and drinking before the collection of EBC. Exhaled air entered and left the chamber through one-way valves and an inlet and outlet, thus keeping the chamber closed. Low temperature inside the condensing chamber throughout the collection period resulted in a cooling down of the samples. The temperature at collection was around 0°C [12]. Patients were instructed to breathe tidally for 10 min with a nose clip. The respiratory rate ranged from 15 to 20 breaths/min. Patients were asked to swallow their saliva periodically and to temporarily discontinue collection if they needed to cough. At the end of collection 1.5- to 3.5-ml aliquots of condensate were transferred to Eppendorf tubes and immediately frozen. Samples were stored at -80°C [13]. The longest period of time of EBC sample storing did not exceed 2 months. The samples were not concentrated or lyophilized prior to measurement. All measurements were performed in a blinded fashion. All samples were run in duplicate. The results (RANTES) could be repeated well [ $CV(\%) = 5-6\%$ ]. We performed a preliminary study in which we measured RANTES in EBC immediately after collection and after 1, 2 and 3 months of storage at -80°C and we did not observe any changes. Therefore, we suggest that RANTES in EBC stored at -80°C remains stable for at least 3 months.

Serum total IgE concentrations and serum ECP were measured using ImmunoCAP™ Technology (Pharmacia Diagnostics,

**Table 1.** Clinical, demographic and laboratory characteristics of the groups of patients studied

|                                               | Patients treated with omalizumab | Patients without omalizumab | Differences between groups of asthma patients, p value |
|-----------------------------------------------|----------------------------------|-----------------------------|--------------------------------------------------------|
| Number of patients                            | 9                                | 10                          |                                                        |
| Sex, F/M                                      | 1/8                              | 3/7                         |                                                        |
| Age, years                                    | 49.3 ± 8.2                       | 52.0 ± 8.6                  | 0.95                                                   |
| Duration of symptoms, years                   | 13.4 ± 8.1                       | 15.3 ± 12.5                 | 0.67                                                   |
| Baseline FEV <sub>1</sub> , % predicted       | 49.2 ± 10.8                      | 51.4 ± 8.8                  | 0.63                                                   |
| Blood eosinophil count, cells/mm <sup>3</sup> | 294.4 ± 283.8                    | 170.3 ± 72.8                | 0.14                                                   |
| Baseline F <sub>ENO</sub> , ppb               | 59.4 ± 46.8                      | 41.0 ± 26.6                 | 0.30                                                   |
| Serum total IgE, kU/l                         | 218 ± 123                        | 156 ± 150                   | 0.36                                                   |
| RANTES (EBC), pg/ml                           | 20.45 ± 2.61                     | 16.27 ± 2.58                | 0.002                                                  |
| ECP (serum), µg/l                             | 25.6 ± 18.4                      | 16.6 ± 6.1                  | 0.16                                                   |
| ICS dose <sup>1</sup> , µg/day                | 1,138 ± 253                      | 1,125 ± 317                 | 0.86                                                   |

Data are presented as medians. RANTES = Regulated upon activation, normal T cell expressed and secreted.

<sup>1</sup> Inhaled corticosteroids (fluticasone propionate equivalent).

Uppsala, Sweden). The minimum detectable level of ECP was 2.0 µg/l. Blood eosinophil count was measured using a hematologic analyzer (Coulter Electronics GmbH, Miami, Fla., USA). The concentrations of RANTES (R&D Systems, Wiesbaden-Nordenstadt, Germany) in EBC were determined using an enzyme-linked immunosorbent assay. The minimum detectable level was 2.0 pg/ml.

#### Analysis

Statistical significance was analyzed by using analysis of variance (ANOVA) followed by Bonferroni's *t* test post hoc to determine statistical differences. All values were expressed as means ± SD; *p* < 0.05 were considered significant. The relationship between studied parameters was assayed by correlation. Pearson's linear correlation coefficient was used.

## Results

The characteristics of patients are presented in table 1.

In the group of patients treated with omalizumab, before treatment we observed statistically significantly higher levels of RANTES compared with the group given conventional treatment. There were no statistically significant differences between any other studied parameters in either group of asthma patients before treatment.

In the group of patients treated with omalizumab after 16 weeks of treatment, we found a statistically significant decrease in RANTES levels (before therapy: 20.45 ± 2.61 pg/ml, after therapy: 14.46 ± 3.22 pg/ml, *p* = 0.0008) (fig. 1) and F<sub>ENO</sub> (before therapy: 59.4 ± 46.8 ppb, after therapy: 26.43 ± 18.2 ppb, *p* = 0.01) (fig. 2). We also ob-

served a statistically significant decrease in serum ECP (before therapy: 25.6 ± 18.4 µg/l, after therapy: 10.41 ± 3.45 µg/l, *p* = 0.02) (fig. 3) and blood eosinophil count (before therapy: 294.4 ± 283.8 cells/mm<sup>3</sup>, after therapy: 136.6 ± 114.5 cells/mm<sup>3</sup>, *p* = 0.04) (fig. 4). In this group of patients we also noticed an improvement of spirometric indices (statistically significant increase in FEV<sub>1</sub>: before therapy: 49.2 ± 10.8% predicted, after therapy: 61.5 ± 10.35% predicted, *p* = 0.003).

We did not observe such changes in the group treated with conventional treatment without omalizumab (RANTES – before therapy: 16.27 ± 2.58 pg/ml, after therapy: 15.85 ± 2.41 pg/ml, *p* = 0.42; F<sub>ENO</sub> – before therapy: 41.0 ± 26.6 ppb, after therapy: 34.8 ± 20.16 ppb, *p* = 0.06; serum ECP – before therapy: 16.6 ± 6.1 µg/l, after therapy: 16.3 ± 5.68 µg/l, *p* = 0.48; blood eosinophil count – before therapy: 170.3 ± 72.8 cells/mm<sup>3</sup>, after therapy: 161.6 ± 80.5 cells/mm<sup>3</sup>, *p* = 0.97; FEV<sub>1</sub> – before therapy: 51.4 ± 8.8% predicted, after therapy: 52.6 ± 6.5% predicted, *p* = 0.37).

In the group of patients treated with omalizumab we revealed a statistically significant correlation between the decrease in RANTES and a decrease in F<sub>ENO</sub> after therapy. We also observed a correlation between the decrease in F<sub>ENO</sub> and a decrease in ECP or blood eosinophil count and between the decrease in ECP and a decrease in blood eosinophil count after therapy (table 2). Such correlations we did not observe in the group of patients treated without omalizumab (table 3).

**Fig. 1.** Concentrations of RANTES in EBC before the treatment and subsequent changes observed after 16 weeks in both groups of asthmatic patients (with and without omalizumab therapy).

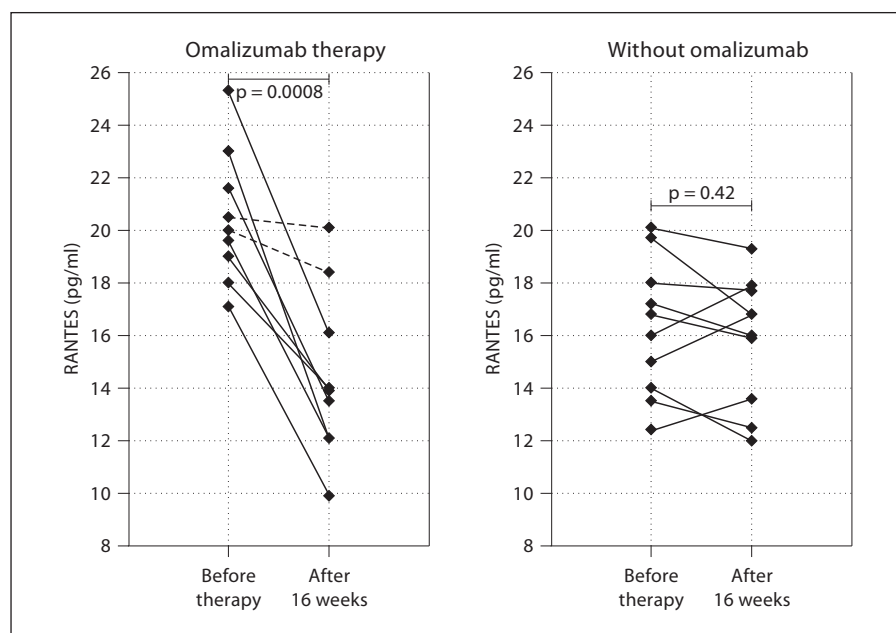
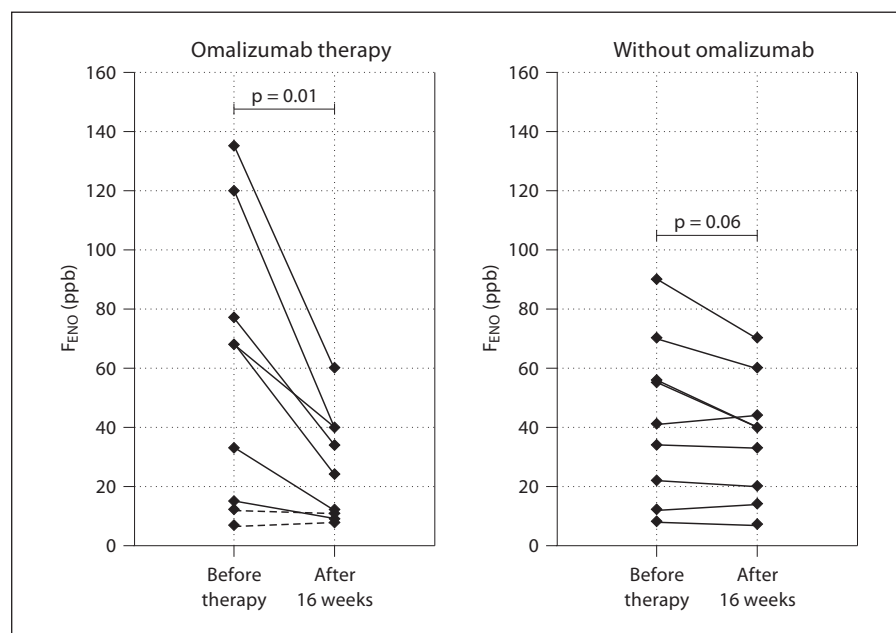


**Fig. 2.** Changes in  $F_{ENO}$  levels observed before and after 16 weeks in the groups of asthmatic patients treated with and without omalizumab.



Omalizumab treatment was well tolerated; we did not observe adverse events. On the basis of a lack of clinical improvement, the Asthma Control Test score or the night and day asthma symptom score and on the basis of a lack of a decrease in daily consumption of asthma rescue medication after 16 weeks of therapy, 2 patients were recognized as nonresponders. The results obtained in these patients are presented in figures 1–5 using an interrupted line.

## Discussion

IgE plays a crucial role in allergic asthma. Binding of IgE to high-affinity ( $Fc\epsilon RI$ ) or low-affinity receptors ( $Fc\epsilon RII$ ) on several cell types and subsequent exposure to an allergen initiate an inflammatory cascade resulting in an induction of cell activation and the release of inflammatory mediators, which contribute to acute and chronic symptoms of asthma [14, 15].

**Fig. 3.** Concentrations of serum ECP before the treatment and subsequent changes observed after 16 weeks in both groups of asthmatic patients (with and without omalizumab therapy).

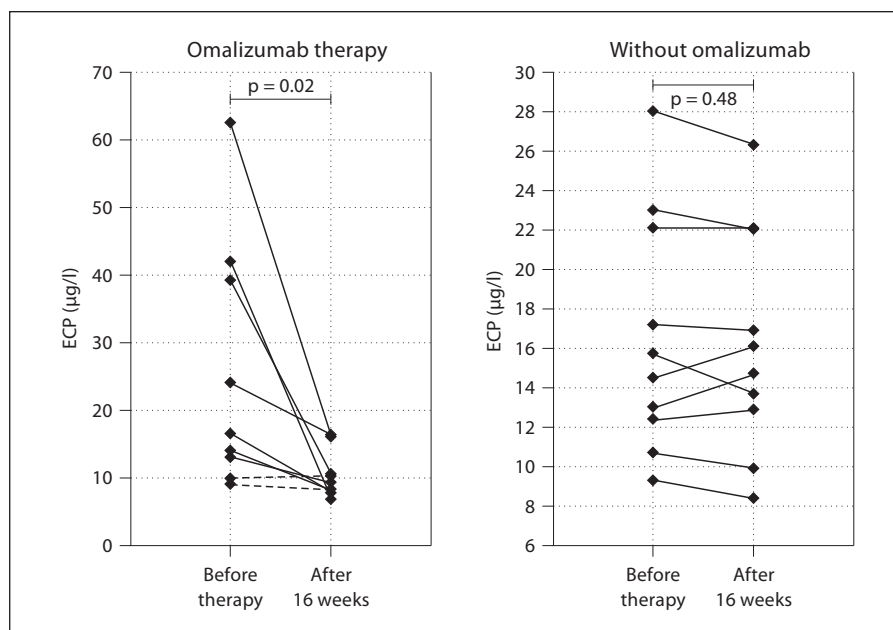
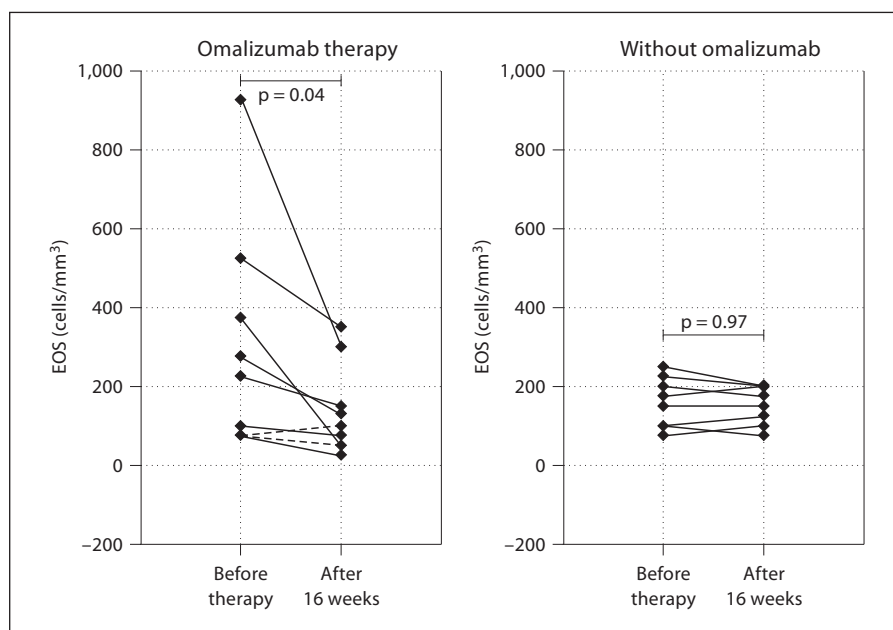


**Fig. 4.** Blood eosinophil count (EOS) in both groups of asthmatic patients (with and without omalizumab therapy) before the study and subsequent changes observed after 16 weeks of treatment.



The key cell, activated by IgE, is the mast cell. Mast cells have been closely associated with cumulating inflammatory cells, mucosal inflammation, endothelial activation, and bronchial hyperresponsiveness, through the release of cytokines such as Th2 cytokines, including IL-4 and IL-5 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [16].

The mechanisms underlying the clinical efficacy of omalizumab are not fully understood; however, the spectrum of anti-inflammatory actions of anti-IgE treatment

extends between the limitation of IgE action on effector cells, such as mast cells and basophils, and the inhibition of the accumulation of inflammatory cells in the lungs as well as a reduction in the release of Th2 cytokines [17].

The recognition of other immunological mechanisms responsible for the effect of omalizumab treatment on the limitation of allergic inflammation, especially connected with eosinophilic inflammation, seems to be very interesting. In published reports so far, there is no data con-

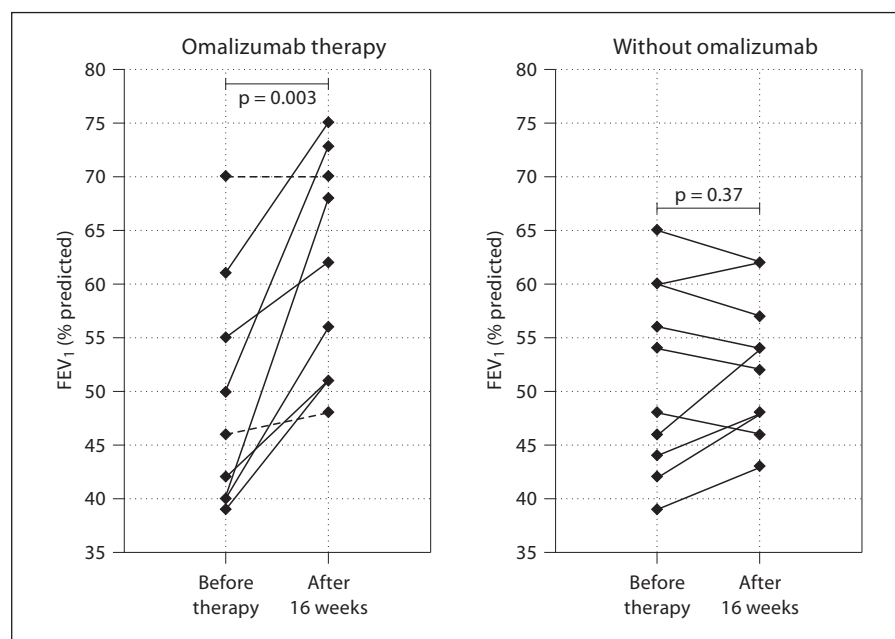


**Fig. 5.** Improvement in FEV<sub>1</sub> observed after 16 weeks of omalizumab treatment and no changes in the group of patients treated without omalizumab.

**Table 2.** Correlations between changes in studied parameters after 16 weeks of therapy in the omalizumab-treated group

| Studied parameters           | F <sub>ENO</sub>       | ECP                  | EOS                  | FEV <sub>1</sub>      |
|------------------------------|------------------------|----------------------|----------------------|-----------------------|
| RANTES (pg/ml)               | r = 0.94<br>p < 0.0001 | r = 0.49<br>p = 0.17 | r = 0.59<br>p = 0.09 | r = -0.63<br>p = 0.07 |
| F <sub>ENO</sub> (ppb)       |                        | r = 0.63<br>p = 0.04 | r = 0.69<br>p = 0.03 | r = -0.55<br>p = 0.12 |
| ECP (μg/l)                   |                        |                      | r = 0.79<br>p = 0.01 | r = -0.46<br>p = 0.2  |
| EOS (cells/mm <sup>3</sup> ) |                        |                      |                      | r = -0.52<br>p = 0.13 |

EOS = Blood eosinophil count.

**Table 3.** Correlations between changes in studied parameters after 16 weeks of therapy in the group of patients without omalizumab

| Studied parameters           | F <sub>ENO</sub>    | ECP                 | EOS                  | FEV <sub>1</sub>      |
|------------------------------|---------------------|---------------------|----------------------|-----------------------|
| RANTES (pg/ml)               | r = 0.2<br>p = 0.57 | r = 0.2<br>p = 0.57 | r = 0.45<br>p = 0.18 | r = 0.21<br>p = 0.56  |
| F <sub>ENO</sub> (ppb)       |                     | r = 0.4<br>p = 0.24 | r = 0.58<br>p = 0.08 | r = 0.29<br>p = 0.4   |
| ECP (μg/l)                   |                     |                     | r = 0.6<br>p = 0.05  | r = -0.38<br>p = 0.26 |
| EOS (cells/mm <sup>3</sup> ) |                     |                     |                      | r = 0.02<br>p = 0.95  |

EOS = Blood eosinophil count.

cerning the effect of anti-IgE therapy on the expression of chemokines, including RANTES, in the airways. These chemokines are very important in the development and maintaining of asthmatic, especially eosinophilic, inflammation. The aim of the study was to assess changes in RANTES concentrations in EBC of asthmatics with severe persistent allergic asthma during omalizumab therapy, and to establish the possible correlation of these measurements with other parameters of airway inflammation.

RANTES is a member of the Cys-Cys (CC) chemokine family [18]. It could evoke the activation of eosinophils, T

lymphocytes, basophils, monocytes/macrophages, and mast cells, and induces the secretion of bronchoconstrictive mediators, such as histamine and cysteinyl leukotrienes from basophils, and ECP from eosinophils [18]. RANTES is also recognized as a potent chemoattractant for these immune cells [18]. Taking all previously described effects of RANTES on inflammatory cells, this cytokine could play an important role in inflammatory cell recruitment and the induction of a release of bronchoconstrictive mediators from cells resulting in airflow limitation.

In a study performed by Teran et al. [19], RANTES levels were measured in bronchoalveolar lavage fluid (BAL) of asthmatic patients exposed to an allergen challenge. The concentrations of this cytokine were increased and correlated with BAL eosinophil count. These results suggest that RANTES is involved in the recruitment of eosinophils into the asthmatic airways after an allergen challenge [19].

Cytokine concentrations in EBC are usually quantified by ELISA kits. Several different cytokines have been described to be present in EBC: IL-4, IL-6, IL-10, IL-1 $\beta$  and TNF- $\alpha$  [12]. However, the concentrations of several cytokines are at around the lower limit of detection. EBC collection is a completely noninvasive way of sampling the respiratory tract that can be repeated frequently with short intervals without adverse events with good reproducibility of EBC volume and mediator concentration for several markers: pH, H<sub>2</sub>O<sub>2</sub>, adenosine, and 8-isoprostane [11].

In our previous studies, we found that RANTES levels in EBC in asthmatic patients are increased compared with healthy controls. In subjects with unstable asthma, EBC concentrations of RANTES were significantly higher than in steroid-naïve and inhaled corticosteroid-treated patients with stable disease. The results of this study suggest that measurements of RANTES in the EBC could be a useful diagnostic tool for the assessment of inflammation, disease severity, and response to treatment in asthmatic patients [20].

Tillie-Leblond et al. [21] demonstrated that high levels of CC chemokines (including RANTES) are present in BAL from patients with severe asthmatic exacerbation; these concentrations are increased compared with that of patients with mild asthma. The authors conclude that CC chemokines could play a role not only in eosinophil recruitment, but also in other inflammatory processes associated with severe asthma.

In this study we have found that, in patients with severe asthma, anti-IgE therapy with omalizumab statistically significantly decreases the concentration of RANTES in EBC. This decrease significantly correlates with the decrease in F<sub>ENO</sub> levels – a more and more important criterion for the evaluation of airway inflammation [22]. Immunological parameters (including RANTES) were evaluated after the inclusion of patients into the studied groups. In the group of patients treated with omalizumab, statistically significant higher baseline levels of RANTES and higher, but not statistically significant baseline levels of parameters used in eosinophilic inflammation assessment were observed. The authors believe

that this is mainly related to the small groups of patients and higher values in 2–3 patients of the omalizumab-treated group. We suggest that it does not change the essential results and conclusions of the study. The main idea of performing this study with two groups of patients was the evaluation of changes of RANTES in EBC during omalizumab treatment and a possible lack of changes in studied parameters in the group using conventional treatment. The differences in initial values of studied parameters seem to have no effect on observed changes and conclusions.

In patients regarded as nonresponders we did not obtain a decrease in RANTES in EBC or in other studied parameters after 16 weeks of anti-IgE therapy. This could suggest that the beneficial clinical effect of omalizumab treatment may also be associated with downregulation of RANTES in the airways.

Oliver et al. [23] revealed that resting basophils can release RANTES and activation of these cells using anti-IgE increases basophil RANTES production. A trend towards reduced RANTES production during omalizumab treatment was observed, but these differences were not statistically significant.

The current study demonstrates a connection between anti-IgE treatment and RANTES expression in the airways. RANTES is released by many types of cells. The main sources of this chemokine are epithelial cells, endothelial cells, macrophages, T lymphocytes, muscle cells, fibroblasts, eosinophils, platelets, Langerhans cells, and dendritic cells. All these cells are present in the airways and many of them are strongly involved in asthmatic inflammation [18]. On the surface of these cells Fc $\epsilon$ R2, the low-affinity receptor for IgE, is expressed, which plays a minimal role in the biological activity of IgE. The high-affinity receptor for IgE, Fc $\epsilon$ R1, which plays an important role in IgE-mediated allergic reactions, is present on mast cells and basophils [15]. These cells are not a potent source of RANTES, but activated by IgE they can indirectly, by production of many proinflammatory cytokines including TNF- $\alpha$ , stimulate other cells to express and release RANTES. Wang et al. [24] have shown that secretion of RANTES from human bronchial epithelial cells can be significantly increased by TNF- $\alpha$  in a dose- and time-dependent manner. The role of TNF- $\alpha$  in the stimulation of RANTES secretion was confirmed in a study performed by Teran et al. [25]. The authors demonstrated that lung fibroblasts are a major cellular source of RANTES after stimulation by TNF- $\alpha$  and INF- $\gamma$ .

Analysis of the obtained results suggests that anti-IgE therapy with omalizumab, through a decrease in IgE con-

centrations, could reduce activation of inflammatory cells and release of RANTES. This may in turn lead to limiting the inflammatory process (related to eosinophil recruitment) and could be one of the key elements responsible for the beneficial effect of anti-IgE therapy with omalizumab.

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