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ORIGINAL ARTICLE

# Evaluation of vascular endothelial growth factor-A and Endostatin levels in induced sputum and relationship to bronchial hyperreactivity in patients with persistent allergic rhinitis monosensitized to house dust☆

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Vascular remodeling

**Summary**
*Background:* Studies about the pathogenesis of bronchial hyperreactivity (BHR) in patients with persistent allergic rhinitis (PAR) and its relationship with lower airway remodeling are extremely limited.
*Objective:* This study evaluated bronchial vascular remodeling via the measurement of angiogenic factor, vascular endothelial growth factor-A (VEGF-A), and anti-angiogenic factor, Endostatin, and evaluated their relationship with BHR in patients with PAR.
*Methods:* The study group consisted of 30 patients with PAR monosensitized to house dust mites and 14 non-allergic healthy controls. All subjects underwent induced sputum and methacholine (M) bronchial provocation tests. VEGF-A and Endostatin levels were measured by ELISA in induced sputum supernatants.
*Results:* The percentages of eosinophils in induced sputum were significantly increased in patients with PAR compared with healthy controls. There were no significant differences between patients with PAR and healthy controls in terms of levels of VEGF (37.9 pg/ml, min–max: 5–373 pg/ml vs. 24.9, min–max: 8–67 pg/ml, $p = 0.8$ respectively), Endostatin

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(532.5 pg/ml, min–max: 150–2125 pg/ml vs. 644, min–max: 223–1123 pg/ml, $p = 0.2$ respectively) and VEGF/Endostatin ratio (0.057 vs. 0.045, $p = 0.8$ respectively). In addition, there were no significant differences between patients who are BHR positive ($n = 8$), or negative to M ($n = 22$) in terms of levels of VEGF, Endostatin and VEGF/Endostatin ratio and no correlations among value of PD20 to M and levels of VEGF, Endostatin and VEGF/Endostatin ratio.
*Conclusion:* We conclude that VEGF-A and Endostatin did not differ between patients with PAR and healthy controls regardless of BHR to M.


## Introduction

The presence of inflammation and airway *remodeling* are cornerstones in the pathogenesis of asthma.[1,2] Angiogenesis has recently attracted considerable attention as a component of airway *remodeling* in bronchial asthma. One of the key molecules for angiogenesis is VEGF; it is widely expressed within many highly vascularized organs including the lungs and is a potent inducer of endothelial cell growth.[3] Vascular *remodeling* and increased expression of associated growth factors such as VEGF are well-recognized features of asthma.[4,5] Endostatin is a strong endogenous inhibitor of angiogenesis and is produced by various types of cells.[6] Endostatin specifically inhibits endothelial cell growth and migration and directly antagonizes the biological effects of VEGF.[7] The vascular component of *remodeling* is regulated by a balance between *angiogenic* and *anti-angiogenic* factors. However, there are no data regarding the balance of major *angiogenic* and *anti-angiogenic* factors in the lower airways of patients with allergic rhinitis (AR) without concomitant asthma.

AR, which is particularly associated with bronchial hyperreactivity (BHR), is considered as a risk factor for asthma development.[8,9] The mechanism of BHR in AR is not fully understood and it is not known whether the BHR in asthma and AR have the same pathophysiologies. Studies on the pathogenesis of BHR in patients with AR and its relationship with lower airway *remodeling* are extremely limited.[10–13] In our first trial, we evaluated bronchial vascular *remodeling and its* relationship with BHR via measurement of VEGF-A and Endostatin levels in allergic rhinitis patients monosensitized to pollen.[10] In the present study, bronchial vascular *remodeling* parameters and their relationship with BHR were evaluated by measuring the same *angiogenic/anti-angiogenic* factors in patients with persistent allergic rhinitis (PAR).

## Methods

### Subjects

Inclusion criteria for patients with rhinitis were as follows: (1) a history of persistent rhinitis without cough, wheezing, or shortness of breath during natural exposure, (2) positive skin test to house dust mites only, (3) baseline forced expiratory volume in 1 second ($FEV_1$) greater than 80% of predicted value. Pulmonary function tests, Bronchial Provocation Test (BPT) to methacholine (M) and induced sputum were performed. All subjects denied any past or present symptoms suggestive of asthma including intermittent dyspnea, wheezing, or a recurrent cough, and any respiratory infection during the month preceding this study. Control subjects had normal spirometry and airway responsiveness to M ($PC_{20} > 16$ mg/ml), had negative skin prick test to common inhalant allergens, no history of rhinitis, no current or past symptoms suggesting asthma, and no respiratory infection during the month before enrollment. Patients and controls were all nonsmokers and were free of all systemic diseases and malignancies. None had eczema or history of nasal polyposis. None of the patients had previously been treated with immunotherapy. All patients discontinued their medications (nasal steroid and oral antihistamine) at least 1 week before M BPT, but they were allowed to use nasal antihistamine spray if necessary. Patients were classified according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines.[14] The study was approved by Ankara University Medical School's Ethics Committee (Decision No: 152-4759).

### Evaluation of atopy

Skin prick tests were performed by using a common panel, including *D. pteronyssinus*, *D. farinae*, grass, tree, and weed pollens, cat, dog, *Alternaria*, *Cladosporium*, and cockroach allergen extracts (Allergopharma, Stockholm, Sweden). The positive and negative controls used were histamine (10 mg/mL) and phenolated glycerol saline, respectively. A mean wheal diameter of 3 mm or greater than that obtained with the control solution was considered positive.

### Pulmonary function tests and nonspecific bronchial provocation test

Pulmonary function tests (Flowhandy Zan 100 USB, Germany) were performed before sputum induction to determine baseline $FEV_1$. BPT using M was performed between 8:30 and 10:30 AM according to the method described by Cockcroft et al.[15] After inhalation of physiologic saline, patients inhaled doubling concentrations of M

from 0.25 to 16 mg/mL diluted in physiologic saline. The challenge was stopped when the $FEV_1$ decreased by more than 20% from the post saline level or when the highest concentration of M had been administered. The result was expressed as $PC_{20}$ M. The $PC_{20}$ M was calculated from the log concentration response curve by linear interpolation of the two last points. A $PC_{20}$ M of more than 16 mg/mL was accepted as the cutoff point.[16]

### Sputum induction and processing

Sputum induction was performed between 9 and 10 AM according to the method proposed by Pizzichini et al. and slightly modified and adapted according to Pavord et al.[17,18] Sputum induction was applied at least three days after the M BPT. Before inhalation of hypertonic saline solution, all patients inhaled 2 puffs of salbutamol 200 μg (Ventolin™, GSK, UK) administered through a metered dose inhaler and underwent spirometry 10 min later. Then an aerosol of sterile 3% saline solution was generated by an ultrasonic nebulizer (output set at 1 ml/min, Omron NE-U17, Japan) and inhaled for 7 min through a mouthpiece without a valve or nose clip. If the patient could not expectorate at this stage, the concentration of saline was increased gradually according to the decrease in FEV1 measurements from baseline. In case of a less than 10% decrease in $FEV_1$, the concentration of saline was increased from 3% to 4% and then to 5%. Sputum induction was performed three times. After each period of inhalation, patients were asked to rinse their mouths and throats carefully, swallow the water, and blowing the nose before expectoration to minimize contamination with saliva and postnasal drip. They were encouraged to cough deeply at 3 min intervals thereafter. The sputum was collected into a container. The collected sputum was pooled and immediately processed. The volume of the entire sputum sample was determined, and an equal volume of 0.1% dithiothreitol (Sputolysin R; Calbiochem, San Diego, CA, USA) was added. The sputum samples were mixed gently with a vortex mixer and incubated for 15 min at room temperature to ensure complete homogenization. Sputum viability was determined with the trypan blue exclusion method to ensure that viability was adequate and then filtered sputum was centrifuged at $450 \times g$ for 10 min (Rotina 38R Hettich, Germany). The resulting cell pellets were resuspended in phosphate buffer saline. A total cell count was carried out using a hemocytometer and cell concentrations were then adjusted to $1.0 \times 10^6$ cells/ml. Slides were stained with May-Grünwald–Giemsa stain for differential cell counts which were performed by the counting of 400 nonsquamous cells by a cytologist in a manner blind to clinical details. The supernatant was stored at −80 °C (Sanyo freezer MDF-U3086S, Japan) for subsequent assays for VEGF and Endostatin.

### Inflammatory cell counts and angiogenic mediators in induced sputum

Four hundred cells were counted in each slide, and inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) were determined as percentages of the total cells using light microscopy (400×).

Vascular endothelial growth factor (*Human VEGF-A ELISA, Bender MedSystems GmBH, Vienna, Austria*) and Endostatin (Quantikine®, Human Endostatin Immunoassay, R&D system Inc., Minneapolis, USA) were measured by ELISA using a specific ELISA kit according to the manufacturer's instructions in induced sputum supernatant. VEGF-A and Endostatin concentrations were quantitated by comparison with a standard curve generated using recombinant. The detection limits were as follows: VEGF-A 7.9 pg/ml, Endostatin 23 pg/ml. The intra- and inter-assay variabilities were, respectively, VEGF-A 6.8 and 8.3%, Endostatin 6.9 and 7.9%.

### Statistics

Data are expressed as median and min–max. Differences among groups were examined by means of Kruskal–Wallis and Mann–Whitney *U*-tests. The significance of correlations was evaluated by determining the Spearman's rho correlation coefficients. A significance level was taken as 0.05 while testing the hypothesis. Data were analyzed using SPSS v. 11.5 (SPSS Inc., Chicago, IL, USA).

## Results

A total of 42 patients with PAR and 22 healthy controls were included in this study. All patients had severe persistent allergic rhinitis according to the current ARIA classification.[14] Sufficient sputum samples were provided in 30 of the 42 PAR patients (F/M: 21/9, mean age: 31.9 ± 11.4 years) and 14 of the 22 controls (F/M: 5/9, mean age: 30.6 ± 6.3 years). There were no significant differences between patients and healthy controls in terms of age, $FEF_{25-75}$, and $FEV_1$ value, weight of the entire sputum and cell viability, but female gender was significantly higher in the PAR group ($p = 0.049$) than in the controls. Cell viability was >50% in the all subjects. Eight PAR patients, but none of the controls, were positive for $PC_{20}$ M ($PC_{20}$M <16 mg/ml). The patients were divided into two groups according to the presence or absence of BHR. There were no significant differences between patients with or without BHR and controls in terms of sex, age, time of rhinitis symptoms and diagnosis, $FEF_{25-75}$, and $FEV_1$ value, weight of the entire sputum and cell viability (Table 1).

A significantly greater number of eosinophils were found in the sputum of PAR patients compared to the nonallergic controls, their median (min–max) percentage counts being 0.5 (0–7) and 0 (0–0.2) ($p < 0.001$), respectively. No significant differences were observed for other cell types between controls and patients with PAR. The percentages of eosinophils in the induced sputum were significantly increased in PAR patients with BHR compared to PAR patients without BHR and controls ($p < 0.001$). No significant differences were observed for other cell types among the three groups (Table 2). There was no significant correlation between the number of eosinophils and $PC_{20}$ M value.

The median levels of VEGF were not statistically higher in PAR patients than in healthy controls (37.9 pg/ml, min–max: 5–373 pg/ml vs. 24.9, min–max: 8–67 pg/ml, $p = 0.8$ respectively). Similarly, the median levels of Endostatin were not significantly higher in patients with PAR than in healthy controls (532.5 pg/ml, min–max: 150–2125 pg/ml

**Table 1** Patients' demographic, clinical, functional respiratory, and induced sputum data.

| | BHR (+) PAR n: 8 | BHR (−) PAR n: 22 | Controls n: 14 | *P*-Value |
|---|---|---|---|---|
| Sex (M/F) | 6/2 | 15/7 | 9/5 | >0.05 |
| Age, years | 35 ± 4.4 | 30.6 ± 13.2 | 30.6 ± 6.3 | >0.05 |
| Time of rhinitis symptoms, months | 12 (5–12) | 12 (5–12) | – | >0.05 |
| Time of diagnosis, years | 3.6 ± 2.1 | 4.4 ± 2.7 | – | >0.05 |
| Wheal of *D.pteronyssinus*, mm | 4.5 ± 0.5 | 5.3 ± 2.3 | – | >0.05 |
| Wheal of *D. farinae*, mm | 4.7 ± 1.9 | 5 ± 2.1 | – | >0.05 |
| $PC_{20}$ M, mg/ml | 6.2 ± 4.8 | >16 | >16 | |
| $FEV_1$, % | 96.1 ± 10.1 | 98.6 ± 9.9 | 104.1 ± 7,9 | >0.05 |
| $FEF_{25-75}$, % | 84.8 ± 21.3 | 93.1 ± 18.3 | 102.8 ± 22.1 | >0.05 |
| Weight of sputum, gr | 2.2 ± 0.8 | 2.5 ± 1.3 | 3 ± 1.3 | >0.05 |
| Cell viability, % | 83.6 ± 8.6 | 83.5 ± 19.2 | 77.8 ± 16 | >0.05 |

Results are expressed as means ± SD for age, time of diagnosis, wheal of Der *p*, wheal of Der f, $FEV_1$, $FEF_{25-75}$, weight of sputum, cell viability and $PC_{20}$ M values are expressed as geometric means ± SD. Results are expressed as median (min–max) for time of rhinitis symptoms. BHR (−) PAR, pesistent allergic rhinitis patients without BHR; BHR (+) PAR, persistent allergic rhinitis patients with BHR; M, methacholine.

**Table 2** Cell counts in the induced sputum of control and rhinitis subjects with or without BHR.

| | BHR (+) patients with PAR | BHR (−) patients with PAR | Controls |
|---|---|---|---|
| Total cell number/mL of sputum | 1.1 (0.4–3.8) | 1.0 (0.4–2.8) | 1.2 (0.4–3.6) |
| Eosinophils, % (min–max) | 2.9 (0.3–7)* | 0.4 (0–1.6)** | 0 (0–0.2) |
| Macrophages % (min–max) | 62.5 (52–86) | 78 (52–92) | 70.9 (42.5–92) |
| Neutrophils % (min–max) | 30.5 (10–42) | 20 (5.5–46) | 26.7 (7.5–54.8) |
| Lymphocytes % (min–max) | 1 (0–8) | 1.2 (0–3.8) | 1 (0.2–6) |

* Significantly different from the values for control subjects and PAR patients without BHR ($p < 0.001$).
** Significantly different from the values for control subjects ($p < 0.001$). Data are presented as medians, with interquartile ranges in parentheses.

vs. 644, min–max: 223–1123 pg/ml, $p = 0.2$ respectively). The VEGF/Endostatin ratio was not statistically higher in patients with PAR than in healthy controls (0.057 vs. 0.045, $p = 0.8$ respectively). These results show that there was no statistical difference between groups. In addition, there no significant differences between patients who were BHR positive ($n = 8$) or negative to M ($n = 22$) and controls in terms of levels of VEGF, Endostatin and VEGF/Endostatin ratio. There were no correlations among value of PD20 to M and levels of VEGF, Endostatin and VEGF/Endostatin ratio.

## Discussion

This is the first study about the levels of VEGF-A, Endostatin, and the VEGF-A/Endostatin ratio in induced sputum in patients with PAR. The levels of VEGF-A, Endostatin and the ratio of VEGF-A/Endostatin were not significantly different between patients with PAR and healthy controls. There were no significant differences between patients with or without BHR to M and controls in terms of levels of VEGF, Endostatin and VEGF/Endostatin ratio. The only significant difference between patients and controls was the increased number of sputum eosinophils in patients with PAR.

Although the inflammatory pathogenesis of BHR is understood in asthmatic patients, light has not been shed on the precise mechanism of BHR in patients with AR.[11,19,20] In our trial, we demonstrated higher eosinophil numbers in patients with PAR compared to healthy controls independent of BHR, as in previous studies including our first study in allergic rhinitis patients monosensitized to pollen.[10,21–25] Also, the percentage of eosinophils in induced sputum was found to be significantly higher in PAR patients with BHR when compared with those without BHR The presence of sputum eosinophils in our patients seemed to be linked to the presence of both AR and BHR. However, no correlation between $PC_{20}$ M values and eosinophil levels was observed. This may be related to a probable correlation between airway inflammation and other indirect agents of BPT such as adenosine but not M. the limited number of patients with PAR with BHR may be other factor affecting the lack of correlation between $PC_{20}$ M. On the other hand, eosinophils may not be the only inflammatory cells responsible for the development of BHR when we consider the fact that the anti-IL-5 antibody decreased peripheral blood and sputum eosinophil levels but nevertheless had no effect on BHR in asthmatic patients.[26] These findings imply that other pathologies may underlie the main mechanism of BHR apart from eosinophilic inflammation of the lower airways in AR patients, as already shown in asthma.

Previous studies examining bronchial biopsies have ruled out inflammation as the single factor responsible for the development of BHR and have supported structural changes play a role in this process.[27–29] Even though there have been

numerous studies reporting airway remodeling in asthma, only a few of them have researched airway remodeling in patients with AR. In these studies collagen deposition was demonstrated in bronchial biopsies and this was thought to be responsible for RBM thickness in AR patients.[12,13] Some of the drawbacks encountered in these studies included difficulties in technical application, lack of sufficient samples or the inability of samples to mirror the whole lower airway which in turn could make the evaluation of inflammation and lower airway remodeling using bronchial biopsies impractical. In this respect, current research has focused on the use of non-invasive techniques such as induced sputum that could possibly reflect inflammation remodeling of the lower airway. A recent study carried out with induced sputum showed that AR patients have increased VEGF mRNA levels compared to healthy controls.[11] In another recent study conducted with induced sputum, AR patients were found to have significantly higher VEGF levels compared to healthy controls.[25] In these studies, the possibility of angiogenesis in the lower airway tract of non asthmatic patients with AR was indicated.

Although angiogenesis is regulated by a balance of angiogenic and anti-angiogenic factors.[3,30–32] the relative levels of antiangiogenic factors (Endostatin) in the lower airways of patients with AR have only recently been evaluated by our group.[10] This study compared data obtained during the pollen season from patients monosensitized to pollen with or without BHR to M and healthy controls; it was found that the levels of VEGF-A and the ratio of VEGF-A/Endostatin were significantly higher and the level of endostatin was significantly lower in allergic rhinitis patients monosensitized to pollen with BHR.[10] However, contrary to our expectations, in the present trial, neither the parameters of vascular *remodeling* nor their association with BHR could be demonstrated in patients with PAR monosensitized to house dust. We speculate that the reason for this may be multifactorial, such as the duration and intensity of allergen exposure, severity and duration of symptoms. Severity of the rhinitis did not seem to be a factor since all subjects in the current study and almost all subjects in our previous trial had severe rhinitis. VEGF is not only a *remodeling* mediator but also a mediator of inflammation because it has specifically been shown to increase Th2-mediated inflammation.[33] Therefore, in our first trial we speculated that the high levels of VEGF in the induced sputum of patients with monosensitized to pollen during the pollen season might be associated with increased allergic inflammation in this season. However, in the present study, we did not check the duration and/or intensity of dust mite allergen exposure in the homes and/or workplaces of patients with PAR. Evaluation of the relationship between these levels and the results of induced sputum could have yielded more reliable results.

The main limitation of the current study was the indirect evaluation of vascular *remodeling* based on only two growth factors and the absence of direct histopathological studies from the lower airway. However, previous studies found a correlation between measured vascular *remodeling* parameters (VEGF, MMP-9) using induced sputum and parameters measured with bronchial biopsy.[34,35] Although ethical issue and technical concerns seem to be obstacles in widely use of bronchoscopy in routine practice and research settings in patients with allergic rhinitis, future studies with bronchial biopsy and BAL including other potential factors contributing angiogenesis could reinforce our data. Another limitation was the limited number of patients with PAR, particularly cases with BHR to M. Although the mean level of VEGF-A and ratio of VEGF-A/Endostatin were higher in PAR patients than in healthy controls, the differences were not statistically significant. This was the case for the mean level of Endostatin as well. Inability to obtain statistically significant data and the correlation among $PC_{20}$ M value and levels of VEGF-A, Endostatin, and VEGF-A/Endostatin ratio may be related to this numerical restriction and larger patient numbers may lead to more meaningful results.

In conclusion, based on in induced sputum samples, there was eosinophilic inflammation in the lower airway of patients with PAR with more remarkable inflammation in PAR patients with BHR but its correlation with $PC_{20}$ M value could not be demonstrated. However, levels of VEGF-A and endostatin did not differ between patients with PAR and healthy controls regardless of BHR to M. These non-invasive findings should be confirmed in a larger and well defined study population along with systemic biomarkers

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Author contributions

İnsu Yılmaz selected patients for the study, performed sputum induction and wrote the manuscript, Nilüfer Bayraktar performed ELISA measurements. Derya Seçil performed the sputum induction process, Selcen Yüksel performed the statistical analysis and, Zeynep Mısırlıgil was involved in patient selection, Sevim Bavbek designed the study and revised the manuscript. All authors have read and approved the final manuscript.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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