



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[☆]



İ. Yılmaz^{a,*}, N. Bayraktar^b, K. Ceyhan^c, D. Seçil^a, S. Yüksel^d, Z. Mısırlıgil^a, S. Bavbek^a

^a Ankara University, School of Medicine, Department of Chest Diseases, Division of Immunology and Allergic Diseases, Ankara, Turkey

^b Baskent University, Department of Biochemistry, Ankara, Turkey

^c Ankara University, School of Medicine, Department of Pathology, Division of Clinical Cytology, Ankara, Turkey

^d Ankara University, Department of Biostatistics, Ankara, Turkey

Received 25 December 2014; accepted 30 April 2015

Available online 4 June 2015

KEYWORDS

Bronchial hyperreactivity;
Endostatin;
Eosinophil;
Induced sputum;
Persistent allergic rhinitis;
Vascular endothelial growth factor;
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

[☆] The study was approved by the local ethics committee, and written informed consent was obtained. The study was supported by Ankara University Research Fund (project number: 09B3330019).

* Corresponding author.

E-mail address: insu2004@yahoo.com (İ. Yılmaz).

(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.

© 2014 Sociedade Portuguesa de Pneumologia. Published by Elsevier España, S.L.U. All rights reserved.

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 hyper-reactivity (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 (FEV₁) 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 (PC₂₀ > 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 FEV₁. 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 FEV₁ 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 PC₂₀ M. The PC₂₀ M was calculated from the log concentration response curve by linear interpolation of the two last points. A PC₂₀ 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 FEV₁, 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 × 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 × 10⁶ 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, FEF₂₅₋₇₅, and FEV₁ 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 PC₂₀ M (PC₂₀M < 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, FEF₂₅₋₇₅, and FEV₁ 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 PC₂₀ 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 <i>D. pteronyssinus</i> , mm	4.5 ± 0.5	5.3 ± 2.3	–	>0.05
Wheal of <i>D. farinae</i> , 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, FEV₁, FEF_{25–75}, weight of sputum, cell viability and PC₂₀ M values are expressed as geometric means ± SD. Results are expressed as median (min–max) for time of rhinitis symptoms. BHR (-) PAR, persistent 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 PC₂₀ 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 PC₂₀ 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 PC₂₀ 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 PC₂₀ 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.

References

1. Vignola AM, Mirabella F, Costanzo G, Di Giorgi R, Gjomarkaj M, Bellia V, et al. Airway remodeling in asthma. *Chest*. 2003;123:417-22.
2. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest*. 2003;111:291-7.
3. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nat Med*. 1995;1:27-30.
4. Chetta A, Zanini A, Foresi A, D'Ippolito R, Tipa A, Castagnaro A, et al. Vascular endothelial growth factor up-regulation

- and bronchial wall remodeling in asthma. *Clin Exp Allergy*. 2005;35(11):1437–42.
5. Simcock DE, Kanabar V, Clarke GW, O'Connor BJ, Lee TH, Hirst SJ. Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma. *Am J Respir Crit Care Med*. 2007;176:146–53.
 6. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997;88:277–85.
 7. Yamaguchi N, Anand-Apte B, Lee M, Sasaki T, Fukai N, Shapiro R, et al. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. *EMBO J*. 1999;18:441–523.
 8. Greisner WA, Settupane RJ, Settupane GA. Co-existence of asthma and allergic rhinitis: a 23 year follow-up study of college students. *Allergy Asthma Proc*. 1998;19:185–8.
 9. Danielsson J, Jessen M. The natural course of allergic rhinitis during 12 years of follow-up. *Allergy*. 1997;52:331–4.
 10. Yılmaz I, Bayraktar N, Ceyhan K, Seçil D, Yüksel S, Mısırlıgil Z, et al. Evaluation of vascular endothelial growth factor A and endostatin levels in induced sputum and relationship to bronchial hyperreactivity in patients with seasonal allergic rhinitis. *Am J Rhinol Allergy*. 2013;27:181–6.
 11. Sohn SW, Lee HS, Park HW, Chang YS, Kim YK, Cho SH, et al. Evaluation of cytokine mRNA in induced sputum from patients with allergic rhinitis: relationship to airway hyperresponsiveness. *Allergy*. 2008;63:268–73.
 12. Braunstahl GJ, Fokkens WJ, Overbeek SE, KleinJan A, Hoogsteden HC, Prins JB. Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways. *Clin Exp Allergy*. 2003;33(5):579–87.
 13. Bavbek S, Sencer H, Mısırlıgil Z, Beder S, Gurbuz L. Light and electron microscope study in allergic rhinitis patients (ARP) with or without bronchial hyperreactivity (BHR). *J Invest Allergol Clin Immunol*. 1996;6:172–82.
 14. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic rhinitis and its impact on asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy*. 2008;63 Suppl 86:8–160.
 15. Cockcroft DW, Hargreave FE. Airway hyperresponsiveness: definition, measurement and clinical relevance. In: Kaliner MA, Barnes P, Persson CGA, editors. *Asthma: its pathology and treatment*. New York, NY: Marcel Dekker Inc.; 1991. p. 51–64.
 16. Power C, Screenan S, Hurson B, Burke C, Poulter LW. Distribution of immunocompetent cells in the bronchial wall of clinically healthy subjects showing bronchial hyperresponsiveness. *Thorax*. 1993;48:1125–38.
 17. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid phase measurements. *Am J Respir Crit Care Med*. 1996;154:308–17.
 18. Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax*. 1997;52:498–501.
 19. Canbaz P, Uskudar-Teke H, Aksu K, Keren M, Gulbas Z, Kurt E. Nasal eosinophilia can predict bronchial hyperresponsiveness in persistent rhinitis: evidence for united airways disease concept. *Am J Rhinol Allergy*. 2011;25(2):120–4.
 20. Suh DI, Lee JK, Kim JT, Min YG, Koh YY. Bronchial hyperresponsiveness in preschool children with allergic rhinitis. *Am J Rhinol Allergy*. 2011;25(5):186–90.
 21. Foresi A, Leone C, Pelucchi A, Mastropasqua B, Chetta A, D'Ippolito R, et al. Eosinophils, mast cells, and basophils in induced sputum from patients with seasonal allergic rhinitis and perennial asthma: relationship to methacholine responsiveness. *J Allergy Clin Immunol*. 1997;100:58–64.
 22. Polasa R, Ciamarra I, Mangano G, Prosperini G, Pistorio MP, Vancheri C, et al. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J*. 2000;15:30–5.
 23. Semik-Orzech A, Barczyk A, Wiaderkiewicz R, Pierzchała W. Eotaxin: but not IL-8, is increased in upper and lower airways of allergic rhinitis subjects after nasal allergen challenge. *Allergy Asthma Proc*. 2011;32(3):230–8.
 24. Alvarez MJ, Olaguibel JM, García BE, Rodríguez A, Tabar AI, Urbiola E. Airway inflammation in asthma and perennial allergic rhinitis. Relationship with nonspecific bronchial responsiveness and maximal airway narrowing. *Allergy*. 2000;55(4):355–62.
 25. Kristan S, Malovrh M, Silar M, Kern I, Flezar M, Kosnik M, et al. Airway angiogenesis in patients with rhinitis and controlled asthma. *Clin Exp Allergy*. 2009;39:354–60.
 26. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet*. 2000;356:2144–8.
 27. Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol*. 2001;107:295–301.
 28. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, et al. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax*. 2002;57:309–16.
 29. Boulet L-P, Laviolette M, Turcotte H, Cartier A, Dugas M. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest*. 1997;112:45–52.
 30. Asai K, Kanazawa H, Otani K, Shiraishi S, Hirata K, Yoshikawa J. Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. *J Allergy Clin Immunol*. 2002;110:571–5.
 31. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86:353–64.
 32. Goodwin AM. In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc Res*. 2007;74:172–83.
 33. Lee CG, Link H, Baluk P. Vascular endothelial growth factor (VEGF) induces remodelling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med*. 2004;10:1095–103.
 34. Siddiqui S, Sutcliffe A, Shikotra A, Woodman L, Doe C, McKenna S, et al. Vascular remodeling is a feature of asthma and nonasthmatic eosinophilic bronchitis. *J Allergy Clin Immunol*. 2007;120(4):813–9.
 35. Lee KS, Min KH, Kim SR, Park SJ, Park HS, Jin GY, et al. Vascular endothelial growth factor modulates matrix metalloproteinase-9 expression in asthma. *Am J Respir Crit Care Med*. 2006;174(2):161–70.