

Title

Sputum and BAL Clara cell secretory protein and Surfactant protein D levels in asthma

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Short title: Sputum and BAL CC-16 and SPD in asthma

Abstract

Clara cell secretory protein (CC16) is associated with Th2 modulation. Surfactant protein-D (SPD) plays an important role in surfactant homeostasis and eosinophil chemotaxis. We

measured CC16 and SPD in sputum supernatants of 84 asthmatic patients and 12 healthy. 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.12603

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controls. In 22 asthmatics we additionally measured CC16 and SPD levels in BAL and assessed smooth muscle area (SMA), reticular basement membrane (RBM) thickness, and epithelial detachment (ED) in bronchial biopsies. Induced sputum CC16 and SPD were significantly higher in patients with severe asthma (SRA) compared to mild-moderate and healthy controls. BAL CC16 and SPD levels were also higher in SRA compared to mild-moderate asthma. CC16 BAL levels correlated with ED while SPD BAL levels correlated with SMA and RBM. Severity represented a significant covariate for these associations. CC16 and SPD levels are upregulated in SRA and correlate with remodeling indices, suggesting a possible role of these biomarkers in the remodeling process.

Introduction

Patients with severe refractory asthma (SRA) often have remodeling airway changes [1]. Airway smooth muscle mass, smooth muscle hypertrophy and sub-epithelial fibroblasts have been shown to be the strongest predictors of airflow obstruction in SRA [2]. Thickening of the reticular basement membrane due to extracellular matrix deposition is a particularly distinctive feature of SRA [3]. Biomarkers in sputum supernatant have been previously associated with the remodeling process in SRA [4].

Clara cell secretory protein (CC16) modulates Th2 response, as it down-regulates Th2 differentiation of human naive neonatal T cells through dendritic cell mediation [5]. Induced sputum and serum CC16 levels have been shown not to differ significantly between patients with asthma or rhinitis and controls, but without further stratification of asthma according to severity [6,7].

Surfactant proteins SPA and SPD downregulate airway allergic reactions by inducing a Th-2 to a Th-1 response shift [8], whereas SPD in particular inhibits eosinophil chemotaxis and

eotaxin triggered degranulation [9]. SPD specifically interacts with the CD32 (Fc-gamma II) eosinophil receptor, inhibiting eosinophil cationic protein degranulation [10].

The aim of this study was to evaluate CC16 & SPD levels in sputum supernatants of asthma patients of different severity and to investigate any possible correlations with indices of remodeling derived from endobronchial biopsies.

Methods

We recruited 96 subjects, 40 SRA patients, 44 mild to moderate asthmatics and 12 healthy, non-atopic, non-smoking controls. SRA was defined according to American Thoracic Society criteria. Subjects with any other respiratory, malignant, heart, renal, liver or collagen disease as well as subjects with a respiratory tract infection or asthma exacerbation in the past 8 weeks prior to admission were excluded.

Sputum was induced as previously described, using all modifications for safe measurements according to asthma severity [11].

Bronchoscopy was performed for acquisition of bronchoalveolar-lavage (BAL) and six bronchial biopsy specimens were obtained from various sites of the subsegmental carinae bilaterally.

Slides stained for Hematoxyline-eosine, Smooth muscle actin (SMA) and Periodic Acid-Schiff (PAS) stain were analysed with the Image Pro Plus v5.1.2.59 system (Media cybernetics, SilverSprings, USA) for assessing smooth muscle area (SMA) percentage of biopsy area, reticular basement membrane (RBM) thickness and length of epithelial detachment (ED) as a percentage of the total epithelial length.

Subjects also underwent atopy and lung function assessment. Sputum induction for cell count identification and CC16-SPD measurement in sputum supernatants were performed within one week from the initial assessment. Patients in the bronchoscopy group underwent

bronchoscopy within two weeks from their initial assessment and after performing a sputum induction.

For additional methods, see the Methods section of the online Supporting Information

Results

Patients with asthma had significantly higher levels for both CC16 and SPD in sputum supernatants compared to healthy subjects (Figures1A,1B). Patients with SRA had significantly higher sputum levels of CC16 and SPD compared to those with mild to moderate asthma and healthy controls ($p<0.001$ for both comparisons; Table1, Figure1A,1B). Patients with SRA had also significantly higher levels for both CC 16 and SPD in BAL compared to patients with mild to moderate asthma (Table1, Figures1C,1D]. Mild to moderate asthmatics had significantly higher levels compared to healthy subjects for either CC16 or SPD measured in sputum supernatants (Table1, Figures1A,1B).

Patients with SRA presented significantly increased SMA, RBM thickness and length of epithelial detachment in endobronchial biopsies compared to those with mild to moderate asthma (Table1).

Associations of CC16 and SPD

CC16 in sputum supernatant presented a significant positive association with sputum SPD. CC16 in BAL showed a significant positive association with ED. SPD in BAL was positively associated with SMA and RBM. The presence of SRA represented a significant covariate for both CC16 and SPD associations either in sputum and/or in BAL.

Discussion

We have shown that both CC16 and SPD levels in sputum supernatants and BAL are significantly higher in patients with SRA compared to mild to moderate asthmatics and healthy subjects. Recent evidence suggests that Clara cells regulate the immunomodulatory response and airways' remodeling through the production of specific factors such as CC16.

CC16 is secreted by the non-ciliated Clara cells which reside predominantly in the respiratory bronchioles, and by non-ciliated columnar cells of the large and small airways [12]. After airway injury, Clara cells in stem cell niches proliferate and migrate to replenish the injured terminally differentiated epithelial cells [8]. In fact, after alveolar injury, Clara cells can be seen in the alveolus, a process characterized as alveolar bronchiolization [13]. Accordingly, CC16 has been proposed as a biomarker for epithelial cell dysfunction [14]. The positive correlation of increased CC16 levels with the percentage of epithelial detachment in our study supports a possible role of CC16 as a marker of epithelial injury and regeneration. This process is more intense in severe compared to mild asthma and may explain the higher CC16 levels found in induced sputum and BAL of SRA.

SPD is mainly produced by type II pneumocytes, though Clara cells can also produce small amounts of SPD, partly explaining the modest correlation between CC16 and SPD sputum levels in our study. In a bleomycin-induced fibrosis mouse model, alveolar SPD regulates numbers of macrophages and fibrocytes, profibrotic cytokine expression and fibrotic lung remodeling, indicating a close relationship between SPD and airway remodeling [15]. We found increased sputum and BAL SPD levels in SRA patients that correlated positively with SMA and RBM, further supporting a possible role of SPD in the remodeling process.

The increased SPD amounts in the sputum of patients with SRA could reflect an overproduction of SPD by type II pneumocytes, an altered turnover of SPD, or both. Despite evidence linking SPD with eosinophil chemotaxis and alveolar macrophages, no correlation was found between SPD and sputum cellular populations. Nevertheless, eosinophils were increased in the SRA group compared to mild to moderate asthma.

In conclusion, induced sputum and BAL SPD & CC16 levels are increased in SRA. Additionally, BAL SPD & CC16 levels correlate with bronchial remodeling indices. Our

findings warrant further investigation for the evaluation of SPD & CC16 as candidate remodeling markers in this particular phenotype of asthma.

References

1. Trevor JL, Deshane Js. Refractory asthma: Mechanisms, targets, and therapy. *Allergy* 2014;69(7):817-827.
2. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003; 167:1360–1368.
3. Bourdin A, Neveu D, Vachier I, Paganin F, Godard P, Chanez P. Specificity of basement membrane thickening in severe asthma. *J Allergy Clin Immunol* 2007; 119:1367–1374.
4. Tseliou E, Bakakos P, Kostikas K, Hillas G, Mantzouranis K, Emmanouil P, Simoes D, Alchanatis M, Papiris S, Loukides S. Increased levels of angiopoietins 1 and 2 in sputum supernatant in severe refractory asthma. *Allergy* 2012; 67:396–402.
5. Johansson S, Wennergren G, Aberg N, Rudin A. Clara cell 16-kd protein downregulates T(H)2 differentiation of human naive neonatal T cells. *J Allergy Clin Immunol* 2007;120:308-314.
6. de Burbure C, Pignatti P, Corradi M, Malerba M, Clippe A, Dumont X, Moscato G, Mutti A, Bernard A. Uteroglobin-Related Protein 1 and Clara Cell Protein in Induced Sputum of Patients with Asthma and Rhinitis. *Chest* 2007; 131:172–179.
7. Rava M, Tares L, Lavi I, Barreiro E, Zock JP, Ferrer A, Muniozquren N, Nadif R, Cazzoletti L, Kauffmann F, Anto JM, Guerra S. Serum levels of Clara cell secretory protein, asthma, and lung function in the adult general population. *J Allergy Clin Immunol*. 2013 Jul;132(1):230-2.

8. Madan T, Kishore U, Singh M, Strong P, Clark H, Hussain EM, Reid KB, Sarma PU. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest* 2001;107:467-475.
9. von Bredow C, Hartl D, Schmid K, Schabaz F, Brack E, Reinhardt D, Griesse M. Surfactant protein D regulates chemotaxis and degranulation of human eosinophils. *Clin Exp Allergy* 2006;36: 1566-1574.
10. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P, Ohta K, O'Byrne P, Pedersen SE, Pizzichini E, Sullivan SD, Wenzel SE, Zar HJ. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008; 31:143–178.
11. Kips JC, Fahy JV, Hargreave FE, Ind PW, in't Veen JC. Methods for sputum induction and analysis of induced sputum: a method for assessing airway inflammation in asthma. *Eur Respir J Suppl* 1998;26: 9S–12S.
12. Yoneda K. Ultrastructural localization of phospholipases in the Clara cell of the rat bronchiole. *Am J Pathol* 1978;93:745–752.
13. Atkinson JJ, Adair Kirk TL, Kelley DG, Demello D, Senior RM. Clara cell adhesion and migration to extracellular matrix. *Respir Res* 2008; 9: 1
14. Lomas DA, Silverman EK, Edwards LD, Miller BE, Coxson HO, Tal-Singer R; Evaluation of serum CC16 as a biomarker for COPD in the ECLIPSE cohort. *Thorax* 2008; 63: 1058-1063.
15. Aono Y, Ledford JG, Mukherjee S, Ogawa H, Nishioka Y, Sone S, Beers MF, Noble PW, Wright JR. Surfactant Protein-D Regulates Effector Cell Function and Fibrotic Lung Remodeling in Response to Bleomycin Injury. *Am J Respir Crit Care Med* 2012; 185: 525-536.

Figure legends

Figure 1: **A)** CC16 values [ng/ml] in sputum supernatants in patients with asthma [All, Severe refractory (SR), mild to moderate] and healthy subjects, * $p<0.001$ values indicate difference between asthma and healthy subjects, ** $p<0.001$ -values indicate difference between SRA, mild to moderate and healthy subjects, *** $p<0.001$ -values indicate difference between SRA and mild to moderate, ^ $p<0.001$ values indicate difference between SRA and healthy subjects, \$ $p<0.001$ values indicate difference between healthy subjects and mild to moderate. Values are presented as median interquartile ranges. **B)** SPD values [ng/ml] in sputum supernatants in patients with asthma [All, Severe refractory (SR), mild to moderate] and healthy subjects, * $p<0.001$ values indicate difference between asthma and healthy subjects, ** $p<0.001$ -values indicate difference between SRA, mild to moderate and healthy subjects, *** $p<0.001$ -values indicate difference between SRA and mild to moderate, ^ $p<0.001$ values indicate difference between SRA and healthy subjects, \$ $p<0.001$ values indicate difference between healthy subjects and mild to moderate. Values are presented as median interquartile ranges. CC16=Clara cell protein, SPD=Surfactant protein D.

Figure 2: **A)** CC16 values [ng/ml] in BAL in patients with asthma [Severe refractory (SR), mild to moderate], $p<0.001$ values indicate difference between SRA and mild to moderate asthma. Values are presented as median interquartile ranges. **B)** SPD values [ng/ml] in BAL in patients with asthma [Severe refractory (SR), mild to moderate] $p<0.001$ values indicate difference between SRA and mild to moderate asthma. Values are presented as median interquartile ranges. CC16=Clara cell protein, SPD=Surfactant protein D.

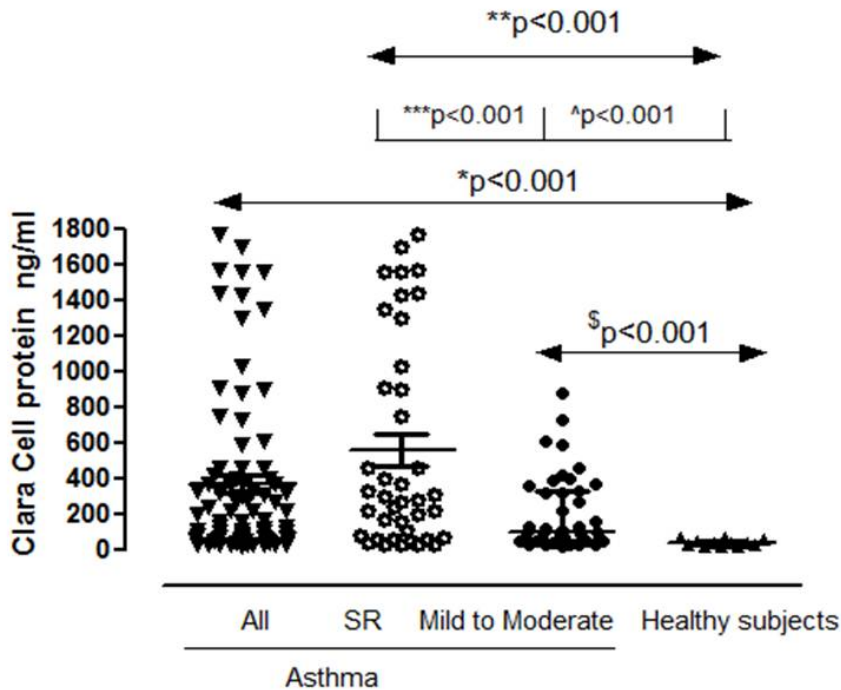
Table 1. Inflammatory variables of study participants.

Variables	SRA n=40 for sputum n=14 for BAL &biopsies	Mild to moderate Asthma n=44 for sputum n=8 for BAL &biopsies	Controls n=12 only sputum data	p value
Induced Sputum				
Cells $\times 10^6$ /ml IS	2.6 (1.4-5) ^{*#}	2 (0.9-3.6) [*]	1.3 $\pm$ 0.9	0.005
Eosinophils (%) IS	7 (3-14) ^{*#}	2 (0-7)	0.5 (0-1)	<0.001
Neutrophils (%) IS	34 (21-47) ^{*#}	25 (19-39)	25 (18-29)	<0.001
Macrophages(%) IS	54 (36-66) ^{*#}	60 (33-73)	68 (58-75)	0.030
Lymphocytes (%) IS	2 (1-2)	0.5 (0.5-1)	0.25 (0-1)	0.580
CC 16 (ng/ml) IS	288 (65-999) ^{*#}	99 (50-324) [*]	33 (21-53)	<0.001
SPD (ng/ml) IS	30.5 (17-63) ^{*#}	8 (4-29) [*]	5 (3.4-7)	<0.001
BAL				
Cells $\times 10^6$ /ml	18(14-21)	19(15-21)		0.621
Eosinophils (%)	10(6.5-17)	3(1-4.5)		<0.001
Neutrophils (%)	4(3-9)	2(2-3.5)		0.036
Macrophages(%)	70 $\pm$ 16	82 $\pm$ 7		<0.001
Lymphocytes (%)	9.5 $\pm$ 5	11 $\pm$ 3		0.187
CC 16 (ng/ml)	277(176-925)	164(90-209)		<0.001
SPD (ng/ml)	2135(1705-2782)	1050(680-1351)		<0.001
Endobronchial biopsies				
SMA %	13.5 $\pm$ 5.0	4.6 $\pm$ 3.0		<0.001
RBM μ m	10.5 $\pm$ 4.5	4.7 $\pm$ 1.5		<0.001
Epithelial detachment %	24 $\pm$ 4	20 $\pm$ 2		0.031

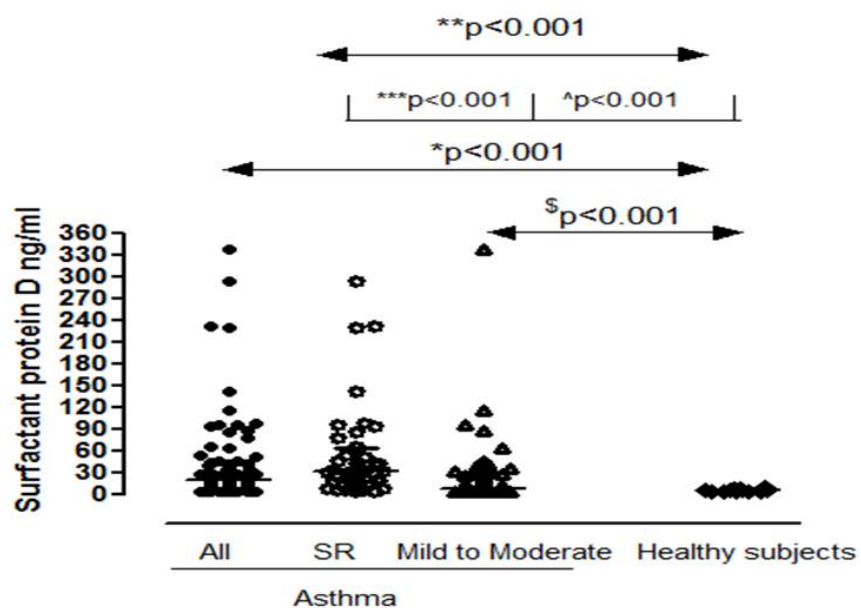
Normally distributed data are presented as mean $\pm$ standard deviation (SD), whereas skewed data are presented as median (interquartile ranges). * Statistically significant difference compared to healthy subjects; # statistically significant difference compared to mild-moderate asthma; Bold numbers indicate significant differences. P-values indicate differences across the 3 groups for IS and across the 2 groups for BAL & biopsies.

Abbreviations: CC16=Clara Cell protein, SPD=Surfactant Protein D, BAL Bronchoalveolar Lavage, SMA= Smooth muscle area, RBM=Reticular basement membrane, ED=epithelial detachment SRA=Severe Refractory Asthma.

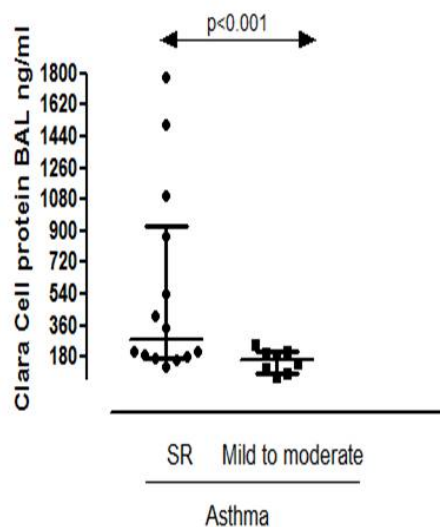
1A



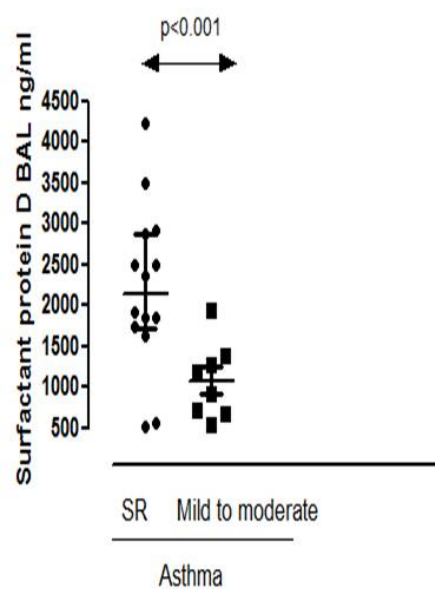
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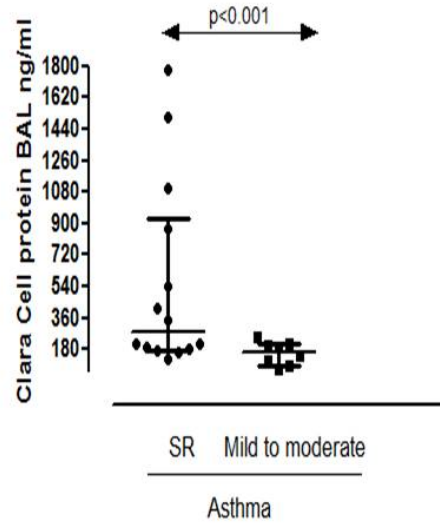
1C



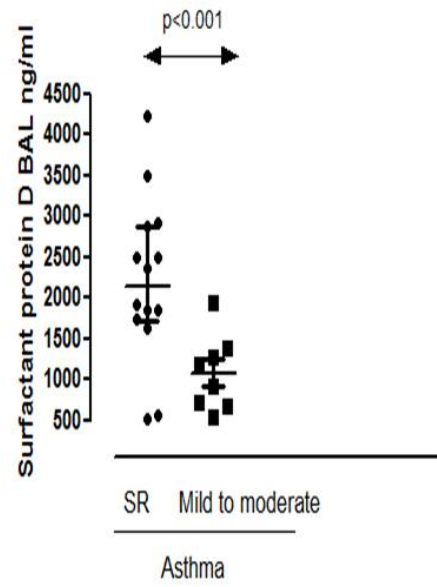
1D



2A



2B



Materials and Methods

Induced Sputum

Sputum was induced as previously described [1], using all the modifications for safe measurements according to the underlying asthma severity [2]. A sample was considered adequate when the patient was able to expectorate at least 2 ml of sputum and the slides contained <10% squamous cells on differential cell counting. Dithiothreitol (DTT) was added in a volume equal to four times the weight of the sputum specimen and it was further diluted with phosphate buffered saline (PBS) in a volume equal to the sputum plus DTT. At least 500 inflammatory cells were counted in each sample. Total cell count was expressed as the number of cells $\times 10^6$. Sputum inflammatory cells were expressed as (%) differential cell counts. Sputum supernatants were kept at -70°C till measurement of the mediators.

Bronchoscopy

Bronchoscopy was performed on an outpatient basis according to published recommendations [3]. All bronchoscopic procedures were performed by two experienced bronchoscopists [SL & PB] using a flexible bronchoscope (Olympus Evis Exera II, Japan). After careful inspection of the bronchial tree, a bronchoalveolar-mini-lavage (BAL) was performed according to the European Respiratory Society Task Force guidelines [4]. Briefly, 120 ml pre-warmed 0.9% saline were instilled into the right middle lobe or lingula in 20-mL aliquots and then gently aspirated. The first aliquot collected was discarded. Only BAL samples with a recovery rate of $\geq 60\%$ were kept for further analysis. Endobronchial biopsy specimens were obtained following BAL collection, from various sites of the subsegmental carinae of the right upper lobe, right lower lobe, left upper lobe. Six bronchial biopsy specimens were

taken from each subject. Tissue was fixed with formaldehyde and embedded in paraffin before cutting.

Morphometry: Slides stained for Hematoxyline-eosine, Smooth muscle actine (SMA) and Periodic Acid-Schiff (PAS) stain were analysed with the Image Pro Plus v5.1.2.59 system (Media cybernetics, SilverSprings, USA). Image analysis of the smooth muscle area (SMA) percentage of the biopsy area was measured at a magnification of x10 on the microscope. Well oriented biopsies containing mucosa and submucosa were used to measure reticular basement membrane (RBM) thickness at a magnification of x20 on the microscope. The length of epithelial detachment (ED) was measured as a percentage of the total epithelial length. Measurements were performed by one blinded pathologist in duplicate.

Lung Function: FEV₁ and FVC were measured as components of flow volume using Master Screen Body (Viasys Healthcare, Jaeger; Hoechberg, Germany) according to American Thoracic Society guidelines [5].

Atopic status

A positive skin prick test to any of twenty common aeroallergens (including mites, grasses, trees, fungus, and domestic animals) was used to confirm atopy.

Mediator assays

Levels of CC16 and SPD were assessed in both sputum supernatant and BAL samples by ELISA according to the manufacturers' instructions (Biovendor R&D Brno, Czech Republic). The minimum detectable levels of CC16 and SPD were 0.02 ng/ml and 0.01 ng/ml respectively.

Study design

On the 1st day all subjects underwent detailed medical history, assessment of the atopic status and lung function tests. Sputum induction for cell count identification and CC16, SPD measurement in sputum supernatants was performed within one week from the initial assessment. Patients in the bronchoscopy group underwent bronchoscopy within two weeks from their initial assessment and after performing a sputum induction.

Statistical analysis

Normally distributed data are presented as mean $\pm$ standard deviation (SD), whereas skewed data are presented as median (interquartile ranges [IR]). Normality of distribution was checked with Kolmogorov–Smirnov test. Statistical comparisons between groups were performed with one-way analysis of variance (ANOVA) for normally distributed data and Kruskal–Wallis tests for skewed data, accompanied by appropriate post hoc tests for multiple comparisons (Bonferroni and Dunn's, respectively). Differences in numerical variables between two groups were evaluated with unpaired t-tests and Mann–Whitney U-tests for normally and skewed data, respectively, whereas comparisons of proportions were performed using chi-square tests. To examine the associations between CC16, SPD, sputum cells, lung function tests and remodeling indices, multivariate linear regression analysis was performed in one model -involving all asthmatic patients and using CC16 and SPD in either sputum supernatants and/or BAL as dependent variables. All linear regressions were performed using as covariates age, gender, body mass index, atopy, duration of the disease, ACT score and presence of SRA and treatment regimens. Data were interpreted as standardized coefficients with 95% confidence intervals. A p-value <0.05 was considered significant. Statistical analysis was performed using SPSS 16.0

(SPSS, Chicago, IL, USA) and GRAPH PAD PRISM 5 (GraphPad Software, San Diego, CA, USA).

References

1. Kips JC, Fahy JV, Hargreave FE, Ind PW, in't Veen JC. Methods for sputum induction and analysis of induced sputum: a method for assessing airway inflammation in asthma. Eur Respir J Suppl 1998;26: 9S–12S.
2. Grootendorst DC, van den Bos JW, Romeijn JJ, Veselic-Charvat M, Duiverman EJ, Vrijlandt EJ, Sterk PJ, Roldaan AC. Induced sputum in adolescents with severe stable asthma. Safety and the relationship of cell counts and eosinophil cationic protein to clinical severity. Eur Respir J 1999;13: 647–653.
3. Jeffery P, Holgate S, Wenzel S. Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment. Am J Respir Crit Care Med 2003; 168: S1–S17.
4. Haslam PL, Baughman RP. Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL. Eur Respir J 1999; 14: 245–248.
5. Standardization of Spirometry, 1994 Update. American Thoracic Society. Am J Respir Crit Care Med 1995;152:1107–1136.