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The profile of respiratory pathogens in induced sputum of elderly and non-elderly asthmatics

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Abstract

Introduction: *Respiratory pathogens are thought to be involved in the pathogenesis and exacerbations of asthma at all ages; however, little is known about the airway microbiome in the elderly.*

Aim of the study**:** *To identify respiratory pathogens in the induced sputum (IS) of elderly asthmatics, and to determine the association between pathogens and the markers of asthma activity.*

Material and methods: *Twenty-nine subjects with stable asthma, 15 above 65 years of age and 14 aged 30-49 years, underwent clinical evaluation, fractional exhaled nitric oxide measurement, and sputum induction. Pathogens were detected by multiplex reverse transcription polymerase chain reaction. The periostin concentration of IS supernatants was measured by enzyme-linked immunosorbent assay. Serum eosinophil cationic protein and total IgE levels were measured by ImmunoCAP.*

Results: *Elderly patients, as compared to non-elderly, had significantly higher eosinophilia in IS, although other markers of eosinophilic inflammation were comparable. Half of the subjects were positive for Haemophilus influenzae. Chlamydophila pneumoniae was found in two subjects. Respiratory viruses were detected in more than 70% of patients. The detection rates and profiles of atypical bacteria and respiratory viruses were similar in both groups. Only in the elderly asthmatics was influenza A positivity associated with lower predicted FVC%, RSV A positivity connected with decreased tIgE concentration, and RSV B positivity related to a lower percentage of lymphocytes in IS.*

Conclusions: *Despite the existence of differences in some clinical and inflammatory characteristics of asthma between elderly and non-elderly asthmatics, the pathogen detection rates in the IS from the two groups are similar.*

Key words: *asthma, elderly, pathogens, induced sputum.*

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Introduction

Ageing is often characterised by a progressive decline in immune response, resulting in increased susceptibility to infections and poor response to vaccination [1]. Clinical observations suggest that lower respiratory tract infections may contribute to the pathogenesis of asthma and are important triggers for asthma exacerbations [2]. Asthma in the elderly seems to be a distinct phenotype with higher severity and exacerbation rates [3], which may be associated with the deterioration of immune function observed in the airways in the aged population [4].

The airway microbiome might have a profound role on the development, persistence, and clinical course of chronic inflammatory diseases like asthma and chronic obstructive pulmonary disease [5]. The presence of respiratory viruses during childhood has been identified as a significant risk factor for asthma in adolescences and adults [6], while similar observations have been made for atypical bacteria [7]. Viruses, such as rhinovirus, respiratory syncytial virus, or parainfluenza viruses, are the most common causative factors for asthma exacerbation [8]. In clinically stable periods of asthma, both viruses and bacteria are detected in the airways [9, 10], but the significance of the persistent presence of those pathogens in the airways remains unclear. While the majority of studies indicate that the detection rate of respiratory viruses in patients with asthma and healthy subjects is similar [10], they have been found to differ with regard to the bacterial composition of the airways [11].

Information on the respiratory pathogens of the lower airways and their relationship with the nature of asthma

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in elderly asthmatics is limited, mostly because of the risk and inconvenience associated with direct and invasive methods of airway lumen sampling. Induced sputum (IS) allows for non-invasive assessment of lower airway inflammation and can be employed to detect respiratory pathogens in the lower respiratory tract. This technique was chosen for the present study, to test the hypothesis that elderly patients with asthma display a different profile of respiratory pathogens in the airways as compared to non-elderly asthmatics, and that this profile may be related to local airway and/or systemic inflammation.

Material and methods

Patients

Twenty-nine clinically stable, non-smoking patients with moderate or severe persistent asthma, 15 elderly (above 65 years of age) and 14 non-elderly (aged 30-49 years), were included to the study. None of the individuals demonstrated any symptoms of infection or had used antibiotics or oral corticosteroids for six weeks prior to the procedure. The level of asthma control was established according to Global Initiative for Asthma (GINA) 2011 guidelines [12] and assessed by the Asthma Control Test (ACT) [13]. Severe asthma was defined according to the American Thoracic Society (ATS) Workshop 2000 criteria [14]. Spirometry was performed according to European Respiratory Society (ERS) standards [15], using a PNEUMO RS spirometer (Abcmed Artmed, Kraków, Poland). The panel of skin prick tests (Allergopharma, Reinbek, Germany) included the following allergens: *Dermatophagoides pteronyssinus, Dermatophagoides farinae*, cat, dog, rabbit, hamster, guinea pig, rat, swine, birch, grass mix, mugwort, plantain, *Alternaria tenuis*, and *Cladosporium herbarum*. A positive result was defined as a wheal of 3 mm or more in diameter. Atopy was diagnosed as the presence of at least one positive skin test.

FeNO measurement

FeNO was measured by using the online single breath method with NIOX (Aerocrine, Solna, Sweden), according to ATS guidelines [16]. The measurement was performed before the sputum induction.

Sputum induction

Sputum induction was performed according to Pin et al. with some modifications [17, 18]. Briefly, patients received 200 µg of salbutamol from a metered dose inhaler followed by inhalation of increasing doses of hypertonic saline solution (3%, 4%, 5%) using an ultrasonic nebuliser UltraNeb (Devilbiss Health Care Inc., Somerset, USA). Patients were asked to expectorate sputum after gargling mouthwash. Spirometry was performed before the procedure, 15 minutes after salbutamol inhalation, and every seven minutes after the start of the saline nebulisation. When their FEV1 decreased by 20% or more, the sputum induction was stopped.

Processing and analysis of the induced sputum

The sputum sample was kept on ice and processed within two hours. Dithiothreitol (DTT) (Sigma Aldrich, St Louis, USA) was added to the selected plaques of sputum and mixed for 15 min on ice. The sample was filtered using a 52-µcm nylon gauze (Surtex, Lodz, Poland). The total cell count was measured using a haemocytometer and centrifuged. The cell pellets were resuspended with phosphate-buffered saline (PBS), and 75 µl of this cell suspension was used to perform the cytospin slides stained with RapiHem solution, a variant of May-Grünwald-Giemsa staining (Aqua-Med, Lodz, Poland). Only smears with less than 50% of non-squamous cells were considered to be adequate for the differential cell counting. On each slide, 300 cells were counted, and the macrophage, lymphocyte, neutrophil, and eosinophil values were expressed as percentages of the total number of inflammatory cells. The supernatants were stored frozen at –80° for further analysis.

Detection of bacteria

Bacterial DNA was extracted from 200 µl of each sputum specimen using a QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, USA), according to the manufacturer's instructions. A qualitative *in vitro* test, Seeplex PneumoBacter ACE Detection (Seegene, Seoul, South Korea) was used for the detection of six pathogens: *Mycoplasma pneumoniae* (MP), *Legionella pneumophila* (LP), *Streptococcus pneumoniae* (SP), *Haemophilus influenzae* (HI), *Bordetella pertussis* (BP), and *Chlamydophila pneumoniae* (CP). The multiplex PCR products were visualised by electrophoresis on 2% agarose gel.

Detection of viruses

Viral DNA or RNA was extracted from sputum using a QIAamp® MinElute® Virus Spin Kit (Qiagen), according to the manufacturer's instructions. To synthesise cDNA, reverse transcription was performed using a RevertAid H Minus First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, USA). Each cDNA was subjected to multiplex PCR using a SEEPLEX RV15 ACE Detection Kit (Seegene), according to the manufacturer's instructions. The multiplex PCR detected rhinoviruses (HRV) and enteroviruses (HEV), parainfluenza viruses 1 (PIV1), 2 (PIV2), 3 (PIV3), and 4 (PIV4), influenza viruses A (FluA) and B (FluB), respiratory syncytial viruses A (RSVA) and B (RSVB), coronaviruses (229E/NL63 and OC43), adenovirus (AdV), human metapneumovirus (MPV), and human bocaviruses 1/2/3/4 (HBoV). The multiplex PCR products were visualised by electrophoresis on 2% agarose gel.

Inflammatory markers in serum

The serum concentrations of total IgE (kU/l) were determined by ImmunoCAP Total IgE (Phadia AB, Uppsala, Sweden), and the concentrations of ECP (μg/l) were determined using ImmunoCAP ECP (Phadia AB, Upsala, Sweden), according to the manufacturer's instructions.

Periostin in induced sputum supernatants

Periostin was measured in the IS supernatants using an enzyme-linked immunoassay (USCN Life Science Inc., Wuhan, Hubei, China), according to the manufacturer's instructions.

Statistical analysis

Comparisons between the groups were performed with the non-parametric Mann-Whitney *U* test. Spearman rank correlation was used to evaluate the correlation between variables. Results are expressed as median and 25th to 75th percentiles. The statistical analysis was performed using Statistica (StatSoft, USA); p values < 0.05 were accepted as statistically significant.

The study was approved by the local Bioethics Committee, and all study subjects provided their informed consent.

Results

On average, the elderly patients with asthma had significantly lower respiratory function, as well as less current asthma control according to GINA criteria and a lower ACT score, although both groups demonstrated similar disease severity based on the ATS Workshop 2000 criteria (Table 1). The distribution of inflammatory cells in the induced sputum varied depending on the age: older patients had a higher mean percentage of eosinophils ($p < 0.05$) and lymphocytes ($p < 0.05$), and a lower percentage of macrophages ($p < 0.05$) in IS (Table 2). However, the elderly and non-elderly asthmatics had similar mean FeNO levels and concentrations of such eosinophilic inflammation markers as periostin in IS, ECP, and total IgE in serum (Table 1). There

Table 1. Clinical characteristics of elderly and non-elderly asthmatics

Characteristic	Elderly *n* = 15	Non-elderly *n* = 14	*p*
Female, *n* (%)	13 (86.7)	9 (64.3)	NS
Mean age, year ±SD	70.7 ±3.8	40.6 ±6.8	< 0.05
Atopy, *n* (%)	4 (31)	6 (42.9)	NS
Ever smoker, *n* (%)	8 (53.3)	6 (42.9)	NS
FEV1% pred., mean ±SD	82.2 ±20.4	100.7 ±12.1	< 0.05
FEV1% FVC, mean ±SD	72.6 ±8.1	79.7 ±10.7	NS
Asthma control*			
Controlled, *n* (%)	0	7 (50)	< 0.05
Partially controlled, *n* (%)	7 (46.7)	5 (35.7)	NS
Uncontrolled, *n* (%)	8 (53.3)	2 (14.3)	NS
ACT score, mean	18.1	21.9	< 0.05
Patients with severe asthma**, *n* (%)	3 (20)	2 (14.3)	NS
Current ICS treatment			
Low dose	3 (20)	1 (7.1)	NS
Medium dose	8 (53.3)	8 (57.1)	NS
High dose	4 (26.7)	5 (35.7)	NS
Inflammatory markers			
Serum ECP (μg/l), mean ±SD	11 (5.5)	8.1 (4.3)	NS
Total IgE (kU/l), mean ±SD	67.6 (62.6)	101.9 (95.6)	NS
Periostin in IS (pg/ml), mean ±SD	8.9 (14.4)	8.2 (11.3)	NS
FeNO ppb, mean ±SD	22.7 ±10.4	24.1 ±20.7	NS

**asthma control defined according to GINA [12], ** as defined by ATS Workshop 2000 criteria [14], FEV1% pred. – forced expiratory volume in 1 second % of predicted value, FEV1%/FVC – forced expiratory volume in 1 second to forced vital capacity ratio, ACT – asthma control test, ICS – inhaled corticosteroids, ECP – eosinophil cationic protein, IS – induced sputum, FeNO – fractional exhaled nitric oxide*

Table 2. Inflammatory cell counts in induced sputum of elderly and non-elderly asthmatics (results are presented as medians and interquartile range)

Characteristic	Elderly $n = 15$	Non-elderly $n = 14$	p
Total cell count/g sputum	1.77 (0.64-2.5)	1.54 (0.72-1.8)	NS
% macrophages	43.3 (37-50.4)	55.3 (46-70.3)	< 0.05
Macrophages/g sputum	0.79 (0.27-1.22)	0.77 (0.42-1.16)	NS
% lymphocytes	19 (15-36)	11.1 (3.3-19.3)	< 0.05
Lymphocytes/ g sputum	0,24 (0.08-1.21)	0.11 (0.03-0.32)	NS
% eosinophils	9.5 (6-13)	4.6 (3-6.6)	< 0.05
Eosinophils/g sputum	0.17 (0.04-0.26)	0.04 (0.02-0.11)	< 0.05
% neutrophils	17 (12-33)	24.1 (11-27.6)	**NS**
Neutrophils/g sputum	0.25 (0.1-0.57)	0.36 (0.14-0.48)	NS

was no correlation between the number or percentage of eosinophils in the IS and FeNO or ECP in any group.

The presence of respiratory pathogens in the IS

The molecular technique revealed the presence of *S. pneumoniae* in the IS from all asthmatic patients, both elderly and non-elderly, and *H. influenzae* in half the patients from each group (Table 3). *Chlamydophila pneumoniae* was detected in one subject from the elderly group and one from the non-elderly group. All sputa were negative for *M. pneumoniae*, *Legionella*, and *B. pertussis*.

Respiratory viruses were detected in the induced sputum from the majority of patients in both groups: in 11 (73.3%) elderly and 10 (71.4%) non-elderly individuals. The most commonly detected virus was Flu A followed by RSV A and RSV B (Table 3). No differences were observed in the detection rates of viruses between elderly and non-elderly patients.

Association between the presence of virus in the IS with clinical and inflammatory markers

Neither asthma severity according to GINA or ATS criteria nor current asthma control assessed by the ACT were found to correlate with the presence of respiratory pathogens in the IS of asthmatics patients. In elderly asthmatics, but not in the non-elderly group, a few associations between the presence of specific respiratory viruses in the IS and respiratory function/immunological parameters were found. Flu A-positive patients had lower FVC% predicted than negative ones (81.8% pred. ±19.9 vs. 100.4 % pred. ±12,9; $p < 0.05$). The presence of RSV B was associated with a lower percentage of lymphocytes in the IS (28.7% ±12.6 vs. 5.5 % ±9.5; $p < 0.05$), and the RSV A-positive patients had lower tIgE concentration in the serum than RSV A-negative elderly individuals (19.2 kU/l ±17.7 vs. 85.2 kU/l ±64.1; $p < 0.05$).

Table 3. Respiratory pathogens detected in induced sputum of the elderly and non-elderly asthmatics by molecular techniques

Characteristic	Elderly $n = 15$	Non-elderly $n = 14$	p
S. pneumoniae	15 (100)	14 (100)	NS
H. influenzae	8 (53.3)	7 (50)	NS
FluA	8 (53.3)	6 (42.9)	NS
RSVB	6 (40)	5 (35.7)	NS
RSVA	4 (26.7)	4 (28.6)	NS
C. pneumoniae	1 (6.7)	1 (7.1)	NS
HRV	1 (6.7)	1 (7.1)	NS
AdV	–	1 (7.1)	NS
229E/NL63	–	1 (7.1)	NS

S. pneumoniae – Streptococcus pneumoniae, *H. influenzae – Haemophilus influenzae*, FluA – influenza virus A, RSVB – respiratory syncytial virus B, RSVA – respiratory syncytial virus A, *C. pneumoniae – Chlamydophila pneumoniae*, HRV – human rhinovirus, AdV – adenovirus 229E/NL63 – coronavirus, NS – not significant

Discussion

This study is the first to use the IS technique to compare detection rates of respiratory pathogens in the airways of elderly and non-elderly patients with asthma. The total number of positive samples and the profile of respiratory pathogens detected in the induced sputum by molecular techniques were found to be similar in elderly and non-elderly individuals. Bronchial asthma, although heterogeneous, has been considered to be, on average, more severe in the elderly population and associated with increased exacerbation rates mostly related to respiratory infections [19]. Our elderly patients tended to have, on average, more severe disease, as expressed by lower respiratory function, and less current asthma control over the previ-

ous four weeks, demonstrated by GINA criteria and a lower mean ACT score. In addition, the elderly patients had higher sputum eosinophilia and lymphocytosis levels than the non-elderly patients, suggesting the presence of different types of airway inflammation in senior individuals. Although a previous study documents a tendency toward neutrophilic inflammation in the IS from elderly asthmatics, no correlation between neutrophilia and severity of the disease was found [20]. Another study found older asthmatics with fixed obstruction to have a higher percentage of eosinophils and elevated levels of ECP in the induced sputum compared to their peers with chronic obstructive pulmonary disease (COPD), despite an increased percentage of neutrophils in the sputum [21]. Thus, the increased percentage of eosinophils in the IS of elderly patients in our study may reflect the proposed inflammatory profile in the airway mucosa of this population of asthmatics. On the other hand, mean levels of surrogate markers of the airway eosinophilic inflammation (FeNO, ECP, and sputum periostin) were similar in both groups, not confirming differences in the type of inflammation between groups.

Ageing has been associated with several immune abnormalities in both the innate and adaptive immune systems, and consequently with deceased immune response to infections [1]. In this context, the observation of similar detection rate and profile of respiratory pathogens in the induced sputum is rather unexpected. However, our patients had stable asthma during the sputum sampling, and one cannot exclude that the difference in the microbiome between elderly and non-elderly asthmatics might be seen during the disease exacerbations

Although respiratory pathogens have been detected with increased frequency during asthma exacerbations, they may persist in the airways of asthmatics between exacerbation episodes, and it has been postulated that latent or persistent infection may contribute to the chronicity of the airway inflammation [22]. However, a cross-sectional study by Harju *et al*. [9] reports similar detection rates of respiratory viruses in the IS of patients with clinically stable asthma and healthy controls. Similarly, in a prospective study by Tucharelli *et al*. [23] the detection rates of 14 respiratory viruses were comparable in clinically stable asthmatics and in healthy controls at three time points. Accordingly, in non-bronchoscopic bronchoalveolar lavage (BAL), the percentage of viral detection did not vary between asthmatic children and controls [24]. In contrast, the presence of HRV in the lung tissue samples from stable patients was found to be higher in asthmatics than in healthy controls [25]. Thus, the presence or absence of respiratory pathogens in BAL or induced sputum may not reflect the persistence of infection of the airway mucosa.

Although the detection rate and profile of respiratory viruses in IS was similar in elderly and non-elderly patients with asthma, the presence of pathogens was associated with some clinical characteristics only in older subjects. The presence of Influenza A virus in the airways of stable asthma patients (without any clinical signs of infection) was associated with lower respiratory function and RSV B with a lower percentage of lymphocytes in the IS. In a study by Woś *et al*. [25] the presence of HRV was associated with lower spirometric parameters as compared to HRV-negative subjects. Furthermore, in our study the presence of RSV B was associated with a lower percentage of lymphocytes in the IS and the RSV A with lower total IgE concentration in the serum in elderly individuals; however, the significance of this findings remains unclear.

Our study has several limitations. The groups were relatively small, which may have affected the variability of results. The samples were collected during late autumn and winter, which may be connected with more frequent exposure to respiratory infections. Qualitative RT-PCR did not allow active replication indicating active infection to be distinguished from residuals of viral or bacterial DNA or RNA from past infections. The discrepancies between the positivity rates of the respiratory bacteria and viruses shown in the above-mentioned studies may be related to different methods of obtaining samples and detecting pathogens. Supernatants from induced sputum were used as source material, and multiplex RT PCR assays were used for analysis. Harju *et al*. [9] report higher detection rates of viruses in induced sputum and throat swab samples compared to gelatine-filtered expired air, EBC, or nasal swabs. However, viral identification was not consistent between these methods or during the follow-up period. In a study by Falsey *et al.* [26] the identification of *Bordetella pertussis* in IS differed between supernatants and cells from patients with exacerbation of asthma. Hence, our results should be further confirmed in studies involving larger patient populations and, if possible, more direct airway sampling techniques.

Conclusions

In summary, our findings indicate that, despite some differences in the clinical and inflammatory characteristics of asthma between elderly and non-elderly patients, the microbial composition of IS samples from stable patients is similar.

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The authors declare no conflict of interest.

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