® Postepy Hig Med Dosw (online), 2020; 74: 437-442
e-ISSN 1732-2693

www.phmd.pl
Original Article

Received: 13.12.2020
Accepted: 23.06.2020
Published: 15.10.2020

Cilia proteins CFAP36 and sentan in induced sputum as possible new markers of epithelial damage in obstructive lung diseases: A preliminary study

Białka nabłonka urzęsionego: CFAP36 i sentan w plwocinie indukowanej jako możliwe nowe markery uszkodzenia nabłonka w obturacyjnych chorobach płuc – badanie wstępne

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Asthma and chronic obstructive pulmonary disease (COPD) are the most common chronic respiratory diseases characterized by inflammation in the lower airways and epithelium remodeling. Dysfunction of cilium is related to severe asthma and COPD, the role of cilium proteins in obstructive lung diseases is not known. The aim of the study was to evaluate the concentration of cilia associated proteins: sentan and CFAP36 in induced sputum (IS) of asthma and COPD patients.

Materials/Methods: The study involved 15 patients with asthma, 12 patients with COPD and 17 control subjects (9 non-smoking, 8 smoking) who underwent lung function tests and sputum induction. Sentan, CFAP36, IL-6, IL-8, concentrations were measured in induced sputum supernatants by ELISA.

Results: The level of CFAP36 in induced sputum was elevated in asthma patients and subjects with atopy. Cilium protein levels in sputum were not related to spirometric tests results. Both CFAP36 and sentan concentrations were positively correlated with age. The level of sentan was associated with airway neutrophilic inflammation and active smoking status. CFAP36 concentration was negatively related to cell viability, whereas sentan level was positively related, but only in COPD patients.

Conclusions: The results of our study revealed CFAP36 and sentan as possible new markers of epithelial damage of different origin in obstructive lung diseases.

Keywords: cilium, epithelium, obstructive lung disease, asthma, COPD

GICID 01.3001.0014.4522
DOI: 10.5604/01.3001.0014.4522
Word count: 3 000
Tables: 3
Figures: 3
References: 19

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INTRODUCTION

The airway epithelium protects the lung against the noxious environmental factors by numerous mechanisms, including particulate matter trapping and clearance via the mucociliary escalator. Cilia and their function constitutes an important part of the mucociliary transport apparatus, which is involved in cleaning the mucus produced by Goblet cells [2]. The morphology of the epithelium alters throughout the respiratory tract; secretory and ciliated epithelial cells predominate in the upper airways where 50–80% of epithelial cells lining the airways are ciliated [13]. Proteins produced by cilium are transported through the cilia cells by the intraflagellar transport system [18]. Many ciliary proteins have been identified, but so far, their role remains unknown [1].

Asthma and chronic obstructive pulmonary disease (COPD) are the two most common chronic respiratory diseases with different genetic and environmental determinants, both characterized by an inflammation in the lower airways, which leads to airflow limitation. The pathobiology of asthma and COPD is related to epithelium dysfunction and remodeling. It seems that smoking, bacterial colonization, and Th2 inflammation may contribute to impaired cilia function (reduced ciliary beat frequency [CBF] and wave pattern) [8, 16]. Our previous study showed that differences in gene expression profile of macrophages from induced sputum (IS) in asthma and COPD patients were strongly associated with biological processes, such as cilia function and organization [11]. The expression of structural proteins, such as cilia-associated proteins, is usually evaluated by immunohistochemistry staining of the tissue samples. Minding the important role of cilia associated proteins in the pathobiology of asthma and COPD and the significance of studies with non-invasively obtained material from respiratory tract, we assessed the levels of cilium proteins: sentan and cilia-and flagella-associated protein 36 (CFAP36) in IS of patients with obstructive lung diseases. We assumed that cilia associated proteins in IS may came from damaged airway epithelial cells. Because of different indicators that affect the airway epithelial cell injury in asthma and COPD, we speculated that release of cilia associated proteins may be various in these obstructive lung diseases.

The specific aims of the study were as follows: 1) to evaluate the feasibility of CFAP36 and sentan concentration measurement in IS of asthma and COPD patients 2) to assess the relationship between IS CFAP36 and sentan levels with clinical features of asthma and COPD. For additional evaluation of biochemical features of IS we chose IL-6 and IL-8 as well characterized and measurable in IS mediators associated with obstructive lung diseases.

MATERIAL AND METHODS

Study design

This prospective, cross-sectional study involved 15 patients with stable mild-to-moderate asthma, 12 patients with stable mild-to-moderate COPD and 17 control subjects (9 non-smoking, 8 smoking) who underwent lung function tests and sputum induction. In all patients, the diagnosis of asthma or COPD was established according to the up-to-date (2018) GINA [3] and GOLD [4] guidelines, respectively. As well as steroid naïve (freshly diagnosed) as inhaled corticosteroids (ICS) treated asthma patients were recruited to the study. Throughout the study period, the patients continued treatment as prescribed by their attending physician and inclusion into the study did not involve any modification of the treatment. Exclusion criteria for all asthma and COPD patients were systemic steroid treatment, disease exacerbation or symptoms of respiratory tract infection in the previous 3 months. The following evaluations were performed after patient enrollment: medical history, physical examination, spirometry with flow-volume curve, airway obstruction reversibility test, allergy skin prick tests and sputum induction. Atopy was defined as the presence of at least one positive skin prick test to common allergens, with a diameter of 3 mm or greater than the negative control [14]. The control group comprised healthy volunteers with normal spirometry and no history of obstructive lung diseases. Patients' characteristics are shown in Table 1. The study protocol was approved by the Institutional Review Board (KB/249/2016). An informed consent was obtained from all study participants.

Sputum induction and processing

Sputum induction via ultrasonic nebulizer (ULTRA--NEBTM 2000, DeVilbiss, USA) and processing was proceeded as previously described [12]. The IS smears were stained with May-Grünwald-Giemsa method to assess the percentage of immunological cells, based on the morphology of 300 cells from various fields. The criteria for appropriate IS quality were as follows: less than 50% epithelial cells and more than 300 non-epithelial cells on one slide. The IS cell composition of study participants is shown in Table 2.

Protein concentration measurements

Sentan, CFAP36, IL-6, IL-8 concentrations were measured in IS supernatants by ELISA according to the manufacturer's instructions. ELISA kits technical data are presented in Table 3.

Table 1. Patients characteristics

	Asthma n = 15	COPD n = 12	Controls n = 17	p-value
Gender (f/m)	(7/8)	(5/7)	(12/5)	0.15
Age (years)	49 (30.5–59.5)	61 (57.5–70)	37 (28–45)	<0.001
BMI (kg/m2)	25 (24.05–29.375)	26.7 (24.4–28.05)	24.7 (22.95–26.3)	0.42
Post-bronchodilator FEV1 (% predicted)	87 (80–92)	60 (53.5–78)	102.25 (99.25–107.5)	<0.001
post-bronchodilator FEV1/VC (%)	68 (60.5–73)	52 (51–58)	81.5 (76.5–85.25)	<0.001
Disease duration (years)	2 (1–6)	2.5 (0.8125–3)	N.A.	0.75
Atopy (n)	15	4	5	0.002
Smoking status (n) non-smoker/smoker/ex-smoker	14/1/0	0/9/3	9/8/0	<0.001
Cigarette smoke exposure (pack years)	0	38.5 (32.5–50)	1.8 (0–19)	<0.001
ICS treatment (n)	8	0	0	<0.001
CAT (points)	N.A.	9 (6–12)	N.A.	N.A.
ACT (points)	21 (18–25)	N.A.	N.A.	N.A.

ACT – asthma control test, BMI – body mass index, CAT – COPD assessment test, FEV1 – forced expiratory volume at first second, ICS– inhaled corticosteroids, N.A. – not applicable, VC – vital capacity. The results are presented as median and interquartile range (IQR). The p-value was obtained using the Chi square or Kruskal-Wallis test.

Statistical analysis

Statistical analysis was performed with the use of Statistica 12.0 software package (StatSoft Inc., OK, USA). Results are given as median and interquartile range (IQR). Differences between continuous variables were tested using nonparametric Mann-Whitney U test or Kruskal-Wallis test. Correlations between variables were analyzed with Spearman's rank test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

In almost all patients and control subjects, measurable IS CFAP36 and sentan levels were found. IS CFAP36 concentration was significantly higher in asthma patients (0.356 ng/ml (0.255–0.649)) than in controls (0.201 ng/ml (0.136 –0.283)), but not compared to COPD patients (0.437 ng/ml (0.150–0.702)). In terms of IS sentan level, no differences between asthma, COPD and controls were demonstrated (Fig. 1).

In patients with obstructive airway disease, neither CFAP36 nor sentan levels correlated with spirometry results; in contrast IL-6 and IL-8 levels were inversely related to FEV1(%) ($r = -0.449$, $p = 0.003$, $r = -0.429$, $p = 0.005$) and FEV1/VC ($r = -0.396$, $p = 0.009$, $r = -0.433$, $p=0.004$ for IL-6 and IL-8, respectively).

The analysis that included all study participants demonstrated a significant positive correlation between the age and CFAP36 ($r = 0.352$, $p = 0.024$), sentan ($r = 0.367$, $p = 0.018$), IL-6 ($r = 0.445$, $p = 0.004$) and IL-8 ($r = 0.555$, $p = 0.0002$). The level of CFAP36 was elevated ($p = 0.034$) in the group of atopic subjects (0.356 ng/ml (0.219–0.648)) compared to the group of non-atopic subjects (0.211 ng/ml (0.110–0.404)).

CFAP36 level did not correlate with any of IS cell count. On the contrary, sentan concentration was positively associated with sputum neutrophil ($r = 0.341$, $p = 0.029$) and negatively with macrophage ($r = -0.315$, $p = 0.045$) count in the all study participants. A similar pattern of correlation was found for IL-6 ($r = 0.521$, $p = 0.0004$; $r = -0.501$, $p = 0.0007$, respectively) and IL-8 ($r = 0.388$, $p = 0.012$, $r = -0.438$, $p = 0.004$, respectively). No significant correlations between IS cells and evaluated proteins were observed in smaller asthma or COPD groups. We found that both cilia associated proteins correlated with sputum cell viability, but only in COPD patients: CFAP36 ($r = -0.640$, $p = 0.046$) and sentan ($r = 0.701$, $p = 0.023$) (Fig. 2).

Although only IL-6 (116.26 pg/ml (46.063–198.13) vs 23.05 pg/ml (15.831–43.146), $p = 0.002$)) and IL-8 (1606.26 pg/ml (394.91–2600.0) vs 577 pg/ml (344.185–821.845), $p = 0.041$)) were elevated in IS of smokers compared to non-smokers, only sentan correlated with the number of pack-years in active smokers $r = 0.060$, $p = 0.013$ (Fig. 3).

DISCUSSION

To our knowledge, this is the first study which showed that cilia-associated proteins are measurable in IS in both patients with obstructive airway disease and healthy controls. Our study demonstrated an elevated level of CFAP36 in IS of asthma patients and subjects with atopy. We have not seen any correlation between the level of

Table 2. Induced sputum cell composition in asthma, COPD, and control group

	Asthma n = 15	COPD n = 12	Controls n = 17	p-value
Cell viability (%)	82.5 (66–96)	95 (77–98)	84 (77–86)	0.227
Epithelium (%)	11.5 (2.9–18.7)	4.6 (2.0–7.4)	5.7 (2.0–11.5)	0.271
Macrophages (%)	53.2 (32.0–61.9)	30.5 (23.1–44.4)	54.5 (50.5–71.9)	0.003
Lymphocytes (%)	0.9 (0.0–2.4)	0.0 (0.0–0.0)	1.8 (0.8–2.2)	0.048
Neutrophils (%)	24.8 (15.6–48.1)	51.7 (44.3–67.6)	30.8 (11.8–44.2)	0.006
Eosinophils (%)	4.9 (1.0–12.7)	0.5 (0.0–9.9)	0.0 (0.0–0.9)	0.0003

The results are presented as median and interquartile range (IQR). The p-value was obtained using the Kruskal-Wallis test.

Table3. Technical specification of ELISA kits used in the study

	Producer	Catalog number	Sensitivity
Sentan	EIAab (Wuhan, China)	E14775h	0.312 ng/ml
CFAP36	Abbexa (Cambridge, UK)	abx386328	0.062 ng/ml
IL-6	Becton Dickinson (Franklin Lakes, New Jersey, U.S.)	550799	2.2 pg/ml
IL-8	Thermo Fisher Scientific (Waltham, Massachusetts, U.S.)	88–8086	2 pg/ml

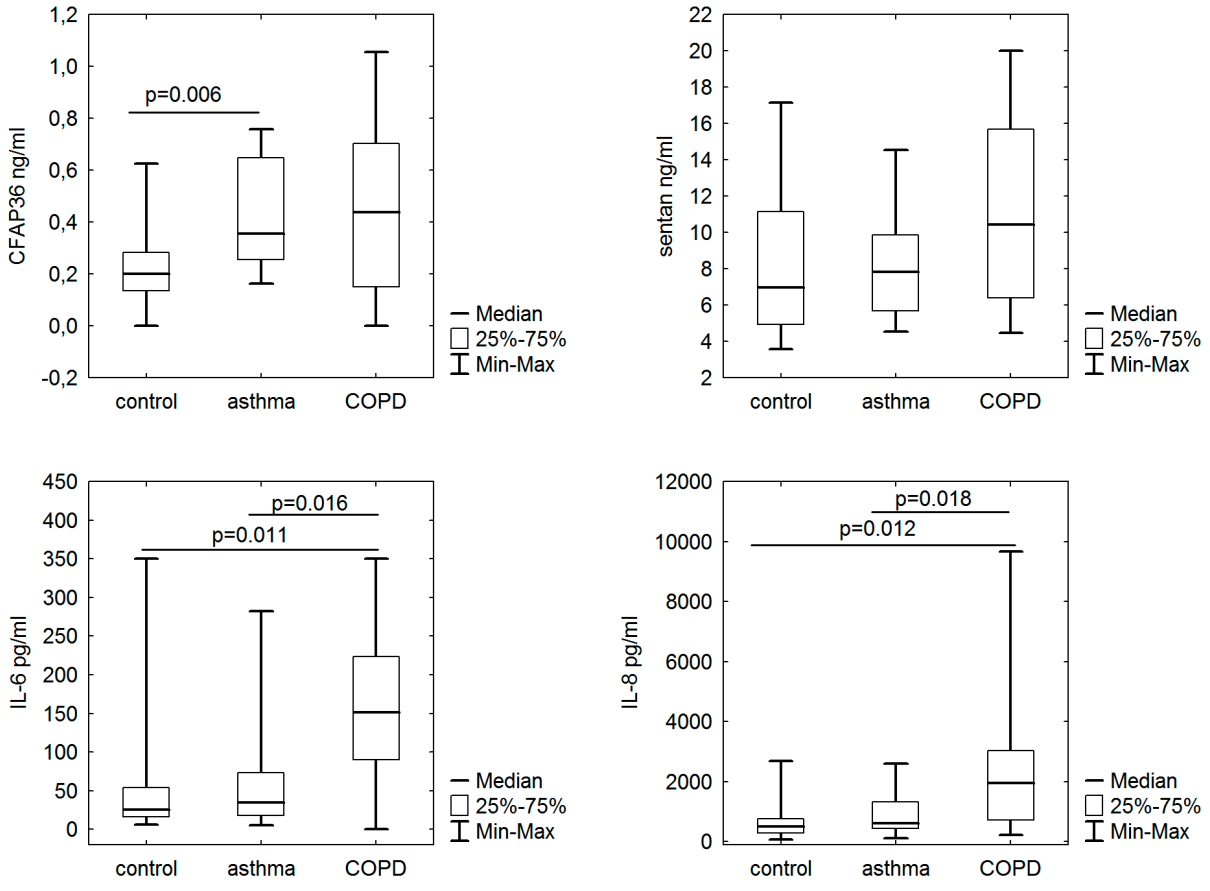

Fig. 1. CFAP36, sentan, IL-6, IL-8 concentration in induced sputum supernatants of healthy controls, asthma, and COPD patients. technique).

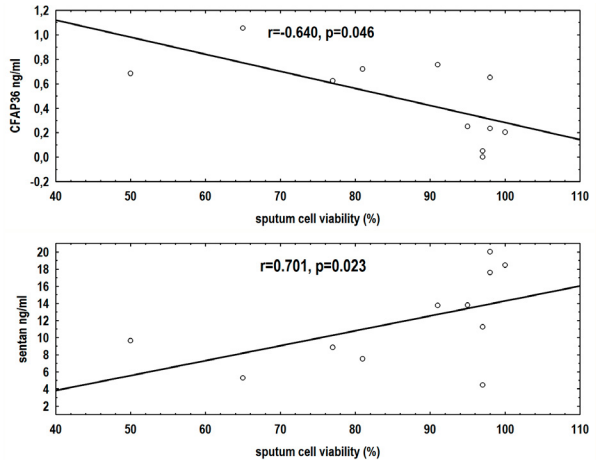

Fig. 2. Correlation between induced sputum cell viability and sentan and CFAP36 concentration in sputum of COPD patients.

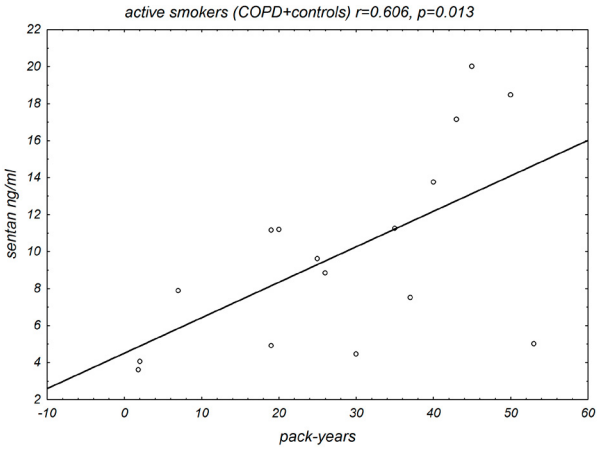

Fig. 3. Correlation between number of pack-years and sentan concentration in induced sputum of active smokers participated into the study (COPD and healthy controls).

airway obstruction measured by spirometry and IS cilium protein levels. We found the association between sentan and neutrophil percentage as well active smoking status. The results of our study showed that CFAP36 is negatively related to cell viability, whereas sentan is positively related, but only in COPD patients.

Literature data concerning the significance of cilium dysfunction in obstructive lung diseases are very scarce. Cilia in patients with moderate and severe asthma were found more dyskinetic and immotile compared to controls; ultrastructural abnormalities of cilia were observed in severe asthma stages [17]. In acute asthma, inhibition of mucin degradation may results in impaired cilia function during exacerbations [7]. The whole exome sequencing identified associations between cilia-related genes and COPD pathobiology [15]. Ciliary dysfunction described as decreased CBF was observed in moderate and severe asthma and COPD patients [14, 16]. The length of cilia was negatively correlated with smoking; moreover, the cilia in COPD patients were shorter than in either smoking or non-smoking controls [6]. The results of our study indicate that different epithelial damage and remodeling processes caused by e.g. inflammatory cells, allergens and smoking could be related to diverse cilium impairment characterized by release of various cilium-associated proteins. The correlation between CFAP36 and asthma or age and between sentan and neutrophil count, age or smoking may suggest that type of airway inflammation (e.g. allergic or cigarette-induced) could impact the IS levels of cilia-associated proteins. The observed positive relationship between sentan, which is cilia structural protein and viability of sputum cells in COPD may also be explained with its association with neutrophilic inflammation. The viability of the cells measured in the study is related to sputum lymphoid but not epithelial cells. Due to neutrophil dominance in COPD, the epithelium damage is positively correlated with increased neutrophil presence in the airways [10]. It is worth noticing that according to literature, corticosteroids can enhance cilia regeneration [9]. In our study, 53% of asthma patients received ICS and we speculate that the treatment could have reduced the CFAP36 level in that group of patients due to recovery of cilia and lowered release of cilia-associated protein.

In contrast to IL-6 and IL-8, we did not observe any correlation between cilia-associated proteins and airflow limitation in asthma or COPD patients. This may be caused by the mild-to-moderate stages of both diseases (slight epithelium damage) of patients recruited to the study. It cannot be excluded that cilia-associated proteins could be a poor marker of obstruction, unrelated to spirometric test results because of other pathological process coexisting in asthma and COPD. In a meta-analysis made by Halbeisen et al., the significant differences between published studies in spirometric indices of patients with primary ciliary dyskinesia were shown, suggesting the vary relation of FEV1 (% predicted) and FVC (% predicted) to disease expression [5].

Our study has some limitations, which are closely related to its preliminary character. Firstly, the study groups were relatively small. Secondly, the study included only patients with mild-to-moderate asthma and COPD, while patients with severe disease were excluded. This was because severe asthma or COPD is commonly associated with FEV1< 50% predicted, which is a contraindication to sputum induction. Bearing in mind that all ciliary dysfunctions observed in the previous studies were attributed to severe asthma or COPD, the lack of correlations between the levels of cilia-related proteins and the severity of airway obstruction can be, at least partially, related to the study group characteristics. Moreover, ICS treatment could have a significant effect on sputum protein concentrations. Thirdly, we did not measure any Th2 cytokines in IS supernatants. The aim of this study was focused on cilium proteins in IS and their relation to clinical features. The Th2 cytokines have a low detectable rate in IS of healthy, mild asthma and mild-to-moderate COPD patients. Finally, the investigated cilia proteins: sentan

and CFAP36 were selected based on their availability in ELISA kits and literature research. We believe that other cilia-associated proteins should be also examined, preferably in a large-scale proteomic screening study.

CONCLUSIONS

We have shown that different cilia-associated proteins are measurable in IS not only in asthma and COPD patients but also in healthy controls. Different patterns of cilia-associated proteins in IS were demonstrated with elevated CFAP36 levels in asthmatics. The results of our study did not reveal any relationship between cilia-associated proteins and impaired lung function, but found the correlation between these proteins and epithelial damage of different etiology. Data from our study are encouraging for further research into cilia-associated proteins in patients with obstructive lung disease pathobiology.

STATEMENT OF ETHICS

All procedures performed in this study were in accordance with the ethical standards of the institutional and /or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by the Institutional Review Board (KB/249/2016). Informed consent was obtained from all study participants.

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Declaration of interest: RK reports personal fees and other from Boehringer Ingelheim, personal fees and other from Chiesi, personal fees and other from AstraZeneca, personal fees from Polpharma, without relevance to the submitted work. KG reports personal fees for lectures and travel expenses from Boehringer Ingelheim, Chiesi, Astra Zeneca, Polpharma and Roche, outside the submitted work. The other authors declare no conflict of interest.