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ORIGINAL PAPER

Evaluation of serum levels of all the transforming growth factor β(TGF-β 1-3) isoforms in asthmatic patients

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ABSTRACT

Introduction: Asthma is a heterogeneous chronic inflammatory disease of the airways. Chronic inflammation leads to changes in the structure and stiffening of the bronchial wall, and irreversible disruption of airflow through the airways. One of the factors researchers consider important in the process of inflammation and fibrosis is the transforming growth factor β (TGF-β). Overexpression of TGF-β in the structural cells of the bronchial tree is thought to translate into excessive secretion of type I and III collagen, fibronectin, tenascin and proteoglycans, as well as inhibition of matrix metalloproteinases and collagenases, promoting the phenomenon of remodeling.

Aim: To evaluate serum levels of all the TGF-β (TGF-β 1-3) isoforms in asthmatic patients, compare the obtained results with those of non-asthmatic subjects, and analyze potential correlations between particular isoforms.

Material and methods: The study included 69 individuals, 41 asthmatic patients and 28 non-asthmatic volunteers recruited from the Department of Allergology and the Allergy and Pulmonology Outpatient Clinic of N. Barlicki University Teaching Hospital No. 1 in Lodz. The participants underwent a full medical examination and spirometry, and blood samples were collected from them for serum TGF-β levels. Individual isoforms, i.e., TGF-β1, TGF-β2 and TGF-β3, were determined by ELISA. The data obtained were then subjected to statistical analysis using Student's *t*-test, the Mann-Whitney *U*-test, Fisher's t-test and Pearson's correlation method.

Results: The obtained results show a significant increase in TGF-β1 and TGF-β2 levels, as well as a slight increase in TGF-β3 concentration among asthmatic patients compared to the general population. Additionally, there occurred a significant association between TGF-β1 and TGF-β2. TGF-β1 and TGF-β2 isoforms were found to be associated with the pathogenesis of asthma. Among the patients, serum TGF-β levels were significantly higher compared to the control group.

Conclusions: The TGF-β1 and TGF-β2 isoforms appear to have a cumulative effect on remodeling processes in the airways.

KEY WORDS

transforming growth factor β, fibrosis, remodeling, asthma.

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INTRODUCTION

Asthma is a heterogeneous disease with three underlying processes: bronchial hyperresponsiveness to specific and non-specific agents, variable and reversible obstruction and chronic inflammation of the bronchial mucosa. The main immune mechanism promoting allergic processes is an imbalance between Th1 and Th2 lymphocytes, in favor of the Th2 fraction. However, it should not be forgotten that in some patients, especially those with more severe forms of the disease, Th1- and Th17-type inflammation may be promoted. Apart from lymphocytes, the immune response cells responsible for initiating and sustaining allergic inflammation are antigen presenting cells (APCs), mastocytes, eosinophils, basophils, macrophages and neutrophil granulocytes, as well as structural cells of the respiratory system such as epithelial cells, myocytes, fibroblasts, myofibroblasts, nerve cells and vascular endothelial cells. Epithelial damage is one of the important causes of bronchial hyperresponsiveness. The influx of eosinophils, neutrophils, macrophages and mastocytes into the airways, and the inflammatory mediators they secrete contribute to contraction of bronchial smooth muscles, breakdown of the extracellular matrix, hyperplasia of mucosal glands and goblet cells. The main mechanisms of obstruction are swelling of the mucosa, excessive mucus secretion and myocardial contraction. Prolonged persistence of an inflammatory process in the airways results in hyperplasia of fibroblasts and myofibroblasts, thickening of the basement membrane of the epithelium, subepithelial fibrosis, deposition of collagens, proteoglycans, fibronectin and tenascin, and promotion of angiogenesis, thus leading to changes in the structure of the bronchial wall, its stiffening and irreversible disruption of airflow through the airways, which is referred to as remodeling [1–4]. One of the factors that researchers consider important in inflammation and fibrosis is the transforming growth factor β (TGF-β).

Depending on the type of tissue, TGF-β regulates the processes of cell synthesis, proliferation, differentiation and apoptosis, and contributes to maintenance of homeostasis in the body. The TGF-β family comprises pleiotropic cytokines produced mainly by fibroblastic and epithelial cells. They stimulate the proliferation of chondroblasts, osteoblasts and neural tissue cells, while they inhibit the growth of epidermal, endothelial and some mesenchymal and hematopoietic cells.

So far, the most thoroughly studied isoform has been TGF-β1 [5, 6]. Its action is associated with immunosuppression of T and B lymphocytes, natural killer (NK) cells, inhibition of expression of MHC class II antigens, but also with chemoattraction and proliferation of fibroblasts, increased synthesis of fibronectin, proteoglycans, type I and III collagen, and inhibition of matrix metalloproteinases [7, 8]. Previous studies have shown that a single nucleotide substitution in the TGF-β promoter region (SNP- C/T at position 509) translated into higher serum concentrations of this cytokine among asthmatic patients, especially in those with severe forms of the disease [9–11].

TGF-β2 is expressed mainly in cells of the adrenal glands, bone marrow, endometrium, colon, liver, lymph nodes, spleen, pancreas, bladder, prostate gland, epithelial and adipose cells. The role it plays mainly involves regulation of the cell cycle. It also fulfills an important function in the morphogenesis processes of the respiratory, cardiovascular, skeletal, digestive and nervous systems, regulation of protein kinase activity and stress-induced cascades [12].

The TGF-β3 isoform is involved in embryogenesis, cell differentiation and controlled apoptosis; it also affects the proliferation and differentiation of primary cells of the internal organs, aortic wall endothelium, perichondrium and chondroblasts, and the bronchial epithelium [13]. Some researchers report that, together with TGF-β1, it induces the expression of fibroblast growth factors, whereas when acting independently, it affects fibroblast proliferation. However, its role in allergic processes has not yet been fully defined [14, 15].

The pleiotropism of TGF-β's action, as well as its ability to exert both pro-inflammatory and immunosuppressive effects, has attracted particular attention from researchers. The anti-inflammatory effect of TGF-β is mainly based on chemoattraction of macrophages and granulocytes to the site of inflammation, differentiation of Th17 lymphocytes and induction of IL-9-producing regulatory T cells. IL-9-producing Treg lymphocytes are a subpopulation of cells that maintain immune homeostasis and control the development of autoimmune diseases. On the other hand, increased production of TGF-β occurs in cells such as eosinophils, neutrophils, mastocytes, macrophages and T and B lymphocytes, whose pro-inflammatory potential is widely known [16]. It is also recognized that overexpression of TGF-β in the structural cells of the bronchial tree: fibroblasts, myocytes, myofibroblasts, the epithelium and the endothelium, translates into excessive

secretion of type I and III collagen, fibronectin, tenascin and proteoglycans, as well as inhibition of matrix metalloproteinases and collagenases. This all leads to increased fibroblast and myofibroblast proliferation, thickening of the basement membrane and subepithelial fibrosis. What arouses interest among scientists is actually the cytokine's potential impact on the remodeling process in asthma, the inhibition of which is one of the main goals to be achieved in the disease treatment.

AIM

The primary objective of this publication was to evaluate serum levels of all the TGF-β isoforms (TGF-β 1-3) in asthmatic patients and to compare them with those found in non-asthmatic volunteers. A secondary objective was to evaluate potential correlations between particular isoforms.

The main research hypothesis of the study is that patients present higher serum concentrations of all the TGF-β isoforms than in the control group. We hypothesize that the isoforms most strongly associated with airway inflammation are TGF-β1 and TGF-β2 which appear to increase cumulatively in asthmatic patients.

MATERIAL AND METHODS

The study included 69 subjects: 41 asthmatics and 28 non-asthmatic volunteers (study group and control group, respectively). The study participants were recruited from the Department of Allergology and the Allergy and Pulmonology Outpatient Clinic of N. Barlicki University Teaching Hospital No. 1 in Lodz, 90-153 Lodz, ul. Kopcinskiego 20.

Study group – inclusion criteria: written informed and voluntary consent to participate in the study, age over 18 years, documented history of asthma, asthma diagnosed according to the GINA (Global Initiative for Asthma) guidelines, absence of other chronic obstructive pulmonary disease, accurate skin prick test (SPT) giving an interpretable result, absence of diseases or medications that affect glucocorticoid metabolism.

Control group – inclusion criteria: written informed and voluntary consent to participate in the study, age over 18 years, no history of respiratory diseases, no history of asthma, no history of therapy with inhaled or systemic glucocorticoids for any cause.

Exclusion criteria: the patient's refusal to give consent at any stage of the study, age under 18 years, pregnancy and breastfeeding, clinical signs of exacerbation when being included in the study or in the preceding 4 weeks, history of a respiratory tract infection in the 4 weeks prior to the study.

STUDY PROTOCOL

The study was approved by the Bioethics Committee of the Medical University of Lodz (RNN/31/14/KE of February 11, 2014).

After providing full information about the study, voluntary written informed consent to participate in the research project was obtained from each subject. On the first appointment, medical interview and physical examination and spirometry were performed [16–18].

MOLECULAR AND BIOCHEMICAL ANALYSIS

Also, at the recruitment appointment, blood samples were taken from all the study participants for molecular-biochemical analysis (4 tubes – 2 EDTA-KE/9 ml tubes and 2 SERUM/9 ml tubes). In the patients subject to bronchial challenge testing (with a specific or non-specific agent), blood samples had been collected before the beginning of the procedure. The collected material was left for complete clotting for 30–60 min, and then centrifuged at 3000 rpm for 10 min using an MPW 223e centrifuge. The resulting serum was portioned into Eppendorf tubes and frozen at –20°C. The serum was thawed at room temperature before testing. The Elabscience® Human TGF-β (TGF-β1, TGF-β2, TGF-β3) ELISA kit was used for determining serum protein concentration. Before use, reagents were prepared according to the manufacturer's instructions, the serum was pre-warmed to 20°C and vortexed. Then, 30 µl of serum was added to 270 µl of Reagent Sample Diluent (1 : 10); next it was lightly vortexed and incubated in a water bath at 80°C for 8 min. After removal, the samples were cooled to 20°C for 5 min. All the reactants at room temperature were mixed thoroughly beforehand. Then, 100 µl of the mixture of serum and reference standard sample diluent (1 : 10) was transferred to the well, mixed thoroughly, and the plate was covered with the provided lid. It was then incubated at 37°C for 90 min. The solution was removed from the wells without washing, 100 µl of Biotinylated Detection Ab was administered immediately and the plate was covered with a sealer. After gentle mixing, they were incubated at 37°C for 60 min. Each well was then aspirated and washed three times with about 350 µl of Wash Buffer. After each step, all the solution was carefully removed from the well. After the last wash, the residual buffer was removed by aspiration. Then, 100 µl of HRP Conjugate was added to each well and covered with a sealer. They were incubated at 37°C for 30 min, and washed repeatedly five times. Next, 90 µl of Substrate Reagent was added to each well; they were covered with sealers and incubated at 37° for 15 min. When a clear gradient was obtained, the reaction was stopped and 50 µl of Stop Solution was added to each well. After the

color changed to yellow, reading was commenced with a plate reader preset to 450 nm and preheated.

STATISTICAL ANALYSIS

The database and statistical analysis were prepared using Statistica 13 software (TIBCO Software inc. 2017, Palo Alto, CA, USA). Selected descriptive statistics were presented using mean and standard deviation for continuous variables. Outliers were converted to limit values. Distribution analysis of continuous variables was made using the Shapiro-Wilk test to confirm normal distribution and the Levene's test to check homogeneity of variance between the groups. The Student's *t*-test for uncorrelated variables was used to compare the two groups, and the Mann-Whitney U test was used in the case of non-normality. Fisher's exact test for "2x2" tables and a χ^2 test with Pearson's correction and Pearson's correlation for continuous variables were used to evaluate the relationship between nominal variables. The analyses were performed for the whole study group and in the individual group, *i.e.,* the asthmatic patients and the non-asthmatic subjects. For all the tests used, $\alpha = 0.05$ was considered as the level of significance (marked with an asterisk *). The results are presented in the tables below, with statistically significant differences marked.

RESULTS

Both study subgroups (the control group – non-asthmatic subjects and the study group – the group of asthma patients) were balanced in terms of sex, BMI, smoking and age (Table 1).

Among all the study participants, non-asthmatic subjects and asthma patients, the highest mean concentrations were observed for the TGF-β1 isoform, where the mean values were 1114.53 ±828.57 pg/ml, while the lowest values were found for the TGF-β3 isoform (84.41 ±53.32 pg/ml). Table 2 shows the mean concentration values, minimum and maximum values, and the median for each TGF-β isoform.

Among the control group, the highest mean concentrations were observed for the TGF-β1 isoform (828.50 ±617.26 pg/ml), while the lowest ones were found for

TABLE 1. Basic clinical parameters of the study and control groups

Parameter	Control group, *N* = 28		Study group, *N* = 41		*P*-value
	n	%	*n*	%	
Gender:					0.24
Male	16	57.1	29	70.1	
Female	12	42.9	12	29.2	
BMI class:					0.34
1	1	3.6	0	0.0	
2	13	46.4	18	43.9	
3	6	21.4	15	36.6	
4	8	28.6	8	19.5	
Smoking:					0.39
Non-smoker	14	50.0	14	34.2	
Non-smoker since min. 6 months	8	28.3	17	41.5	
Active smoker	6	21.4	10	24.4	
	Mean ± SD	Median (min.–max.)	Mean ± SD	Median (min.–max.)	
Age [years]	38.5 ±16.8	32.5 (19.0–68.0)	45.8 ±15.64	50.0 (19.0–71.0)	0.061

TABLE 2. Analysis of the serum level of the TGF-β concentrations in the whole group (*n* = 69, the study and control groups)

Protein	*N*	Mean	Median	Minimum	Maximum	Standard deviation
TGF-β1	69	1114.53	845.80	108.62	3203.30	828.57
TGF-β2	69	871.30	266.81	10.50	3547.59	1067.03
TGF-β3	69	84.41	77.79	4.23	216.29	53.32

the TGF-β3 isoform (68.68 ±52.53 pg/ml). Table 3 shows mean, minimum and maximum concentrations and the median for all TGF-β isoforms in the control group.

Among the asthmatic patients, the highest mean concentration values were observed for the TGF-β1 isoform (1309.87 ±901.67 pg/ml), while the lowest mean values were found for the TGF-β3 isoform (95.16 ±51.76 pg/ml). Table 4 presents mean, minimum and maximum concentration values and the median for each TGF-β isoform in the study group.

When analyzing the correlations between particular TGF-β isoforms, both in the whole group and in each of the two groups separately, statistically significant correlations between the TGF-β 1 and TGF-β2 isoforms were observed in the whole study group (p = 0.000) (Table 5) and in the group of patients (Table 6). No similar trends were observed in the control group (p = 0.07) (Table 7).

DISCUSSION

Despite single reports casting doubt on the role of TGF -β in the pathogenesis of asthma, a vast majority of papers suggest that this cytokine is involved in inflammatory processes occurring in the airways. However, an overwhelming majority of publications have focused researchers' attention on the TGF-β1 subtype, and as a result, we thought it would be interesting to know whether the other TGF-β isoforms may also undergo increased expression (upregulation) in the airways [6, 19, 20]. The main aim of the study was to determine whether there are significant differences in serum TGF-β protein con-

TABLE 3. Analysis of the serum level of the TGF-β concentrations in the control group

Protein	*N*	Mean	Median	Minimum	Maximum	Standard deviation
TGF-β1	28	828.50	725.07	213.49	2946.55	617.26
TGF-β2	28	481.45	204.26	10.50	3336.91	750.71
TGF-β3	28	68.68	48.23	5.36	216.29	52.53

TABLE 4. Analysis of the serum level of the TGF-β concentrations in the study group

Protein	*N*	Mean	Median	Minimum	Maximum	Standard deviation
TGF-β1	41	1309.87	1208.98	108.62	3203.30	901.67
TGF-β2	41	1137.55	741.76	18.57	3547.60	1173.07
TGF-β3	41	95.16	90.08	4.23	216.89	51.76

TABLE 5. Pearson's correlations between the serum level of the TGF-β isoforms

TGF	TGF-β1	TGF-β2	TGF-β3
TGF-β1	$r = 1.00$	$r = 0.63$	$r = 0.23$
	–	$p < 0.001$*	$p = 0.058$
TGF-β2	$r = 0.63$	$r = 1.0000$	$r = 0.12$
	$p < 0.001$*	–	$p = 0.40$
TGF-β3	$r = 0.23$	$r = 0.12$	$r = 1.00$
	$p = 0.058$	$p = 0.34$	–

TABLE 6. Pearson's correlations between the serum level of the TGF-β isoforms in the study group

TGF	TGF-β1	TGF-β2	TGF-β3
TGF-β1	$r = 1.00$	$r = 0.66$	$r = 0.21$
	–	$p < 0.001$*	$p = 0.20$
TGF-β2	$r = 0.66$	$r = 1.00$	$r = 0.013$
	$p < 0.001$*	–	$p = 0.94$
TGF-β3	$r = 0.21$	$r = 0.01$	$r = 1.00$
	$p = 0.20$	$p = 0.94$	–

TABLE 7. Pearson's correlations between the serum level of the TGF-β isoforms in the control group

TGF	TGF-β1	TGF-β2	TGF-β3
TGF-β1	$r = 1.00$	$r = 0.35$	$r = 0.11$
	–	$p = 0.07$	$p = 0.59$
TGF-β2	$r = 0.35$	$r = 1.00$	$r = 0.12$
	$p = 0.07$	–	$p = 0.53$
TGF-β3	$r = 0.11$	$r = 0.12$	$r = 1.00$
	$p = 0.59$	$p = 0.53$	–

centrations between non-asthmatic individuals and asthmatic patients. Our results showed statistically significant higher TGF-β1 and TGF-β2 levels, as well as statistically insignificant, but also higher TGF-β3 isoform concentrations among the asthmatics compared to the healthy population. Similar results were obtained by other researchers, who also found higher TGF-β expression in the bronchial epithelium, alveolar macrophages, vascular endothelium and the Clara cells. Duvernelle *et al.* demonstrated significant differences in TGF-β1 mRNA expression in inflammatory cells (basement membrane eosinophils, alveolar macrophages and lung fibroblasts) obtained from bronchial biopsies in patients with severe and moderate asthma, compared to healthy individuals and those with a mild form of the disease [21–24]. In *in vitro* studies, macrophages from asthmatics secreted higher amounts of TGF-β. The work by Redington *et al.* on TGF-β expression in bronchoalveolar lavage (BAL) showed that it was increased in patients with atopic asthma compared to those with non-atopic asthma [25]. Other studies have shown that TGF-β overexpression in BAL was associated with an increase in the alveolar macrophage count and lipid peroxidation markers related to oxidative stress. Hypotheses regarding associations of TGF-β and oxidative stress on bronchial remodeling concern TGF-β's inhibition of nuclear transcription factor (Nrf, nuclear factor 2), the most powerful antioxidant enzyme in the body. In other reports, an increase in TGF-β1 levels significantly correlated with the number of eosinophils in the sputum of asthmatic patients [24]. Thus, stimulation of pulmonary fibroblast proliferation, alveolar macrophages, and induction of oxidative stress appear to be among the primary mechanisms by which TGF-β1 affects airway remodeling in asthma. It is in contrast with TGF-β2 which, as demonstrated by studies published so far, shows a clear correlation with the expression of subepithelial mucin [25–28]. Interesting data supporting our theses are provided by the study of Chakir *et al.* who prove that TGF-β2 increases not only mucin concentration, but also collagen concentration in fibroblast cells. They showed that among patients with asthma, TGF-β2 concentrations are higher compared to healthy individuals, and that TGF-β2, together with IL-11 and IL-17, is involved in the promotion of collagen I and III production [29]. These are interesting observations considering prevention of fibrosis and subsequent airway remodeling.

In our study, we observed an increase in TGF-β3 concentrations among the asthmatic patients, compared to the non-asthmatic controls; however, these observations were not statistically significant. In previous studies, the role of TGF-β3 has not been fully established yet. Some researchers have observed expression of TGF-β3 isoforms in the bronchial epithelium, but no longer in endothelial and mesenchymal cells. Some researchers have postulated an association of TGF-β3 with the development of asthma with sensitization to Dermatophagoides, but increased mucus and collagen secretion has only been observed in mouse models, and has not been proven in human cell studies. In the light of the current knowledge, it seems that TGF-β3 has a rather marginal role in the pathogenesis of asthma [14, 30].

A very important observation of our study is the relationship between serum concentrations of TGF-β1 and TGF-β2 isoforms. A statistically significant correlation was found for both our whole study population and for the asthmatic patients. We did not observe similar trends in the control group. To support our results, we used the work of Wen *et al.* who, based on human pulmonary fibroblast lines, demonstrated that all the three TGF-β isoforms strongly induced mRNA expression for TGF-β1 and TGF-β2, and, in addition, TGF-β1 strongly stimulated TGF-β2 expression. TGF-β3, on the other hand, had no effect on enhancing TGF-β2 expression, only on TGF-β1 [31]. Another study on which we based our hypotheses was the work of Larkin *et al.*, which examined the expression of TGF-β1 and TGF-β2 in bronchial brushing samples. They showed that the TGF-β2/TGF-β1 mRNA ratio was elevated in asthmatics compared to controls. In the above experiment, no significant differences were observed between the TGF-β1 levels in the patients, compared to the non-asthmatic population, while differences close to statistical significance were observed for

the TGF-β2 isoform in the asthmatic group; however, this was a study with a much smaller number of study subjects [32]. In the publications by Chu *et al.,* respiratory epithelial cells of patients with asthma had significantly higher expression levels of TGF-β2 as compared to TGF-β1. It also seems interesting that TGF-β2 can be induced by IL-13, whose pro-inflammatory potential in asthma is, in fact, well known [28, 33]. After all, the use of antibodies that block the receptor for this cytokine is common in the treatment of the most severe forms of the disease. In our opinion, it is worth noting that IL-13 also stimulates type I collagen production by airway fibroblasts in a matrix metalloproteinase-dependent manner, as well as TGF-β1 -dependant one [34]. Thus, it may be hypothesized that when both TGF-β cytokines act together on the airways, their pro-inflammatory and fibrosis-promoting potential will be higher.

One of the main characteristics differentiating our study from those mentioned above was the evaluation of the TGF-β isoforms based on their serum concentrations, rather than in bronchial biopsy specimens, bronchoalveolar lavage or sputum. We compared our results with the publications of the Manuyakorn team of researchers, who also studied serum TGF concentrations, on a similarly sized pediatric population. They obtained the same results indicating higher TGF-β1 concentrations among patients, compared to healthy individuals [35]. Data in support of our hypothesis are also provided by the work of Panek *et al.* which assessed serum TGF-β mRNA in asthmatic patients and healthy volunteers. Expression values among asthmatics were significantly higher and varied depending on the severity of the disease [11, 36, 37]. Evaluation of TGF-β in blood serum only would seem to be a significant limitation of the present study, however, it was a deliberate effort to seek indirect, non-invasive and readily available markers for assessing the severity of inflammatory processes in the airways. We have so far chosen not to perform BAL or bronchial biopsy due to the invasiveness of this test and the reluctance to undergo bronchoscopy among patients, which could potentially narrow the study group. Nevertheless, we realize that this would significantly increase the value of the work. At present, having found statistically significant differences in blood TGF-β concentrations in asthmatic patients compared to healthy subjects, we wish to continue our research and extend it to include bronchial tissue tests, with simultaneous evaluation of serum concentrations. We believe that assessing the correlation of the expression of individual TGF-β isoforms in the bronchioles and serum will provide new and interesting data that could answer the question of whether TGF-β can potentially be considered as an indirect marker of airway remodeling in patients with asthma [38]. The search for markers that are readily available, inexpensive and non-invasive is a trend that has occurred in medicine for many years. Peripheral eosinophilia is commonly used as a marker of the intensity of the inflammatory process, based on which advanced biologic therapy is applied in asthmatics with the most severe form of the disease [39–44].

CONCLUSIONS

The three TGF-β isoforms are not biologically equivalent. From the point of view of airway inflammation, the most relevant isoforms appear to be TGF-β1 and TGF-β2. In the light of studies that suggest higher TGF-β concentrations among patients with atopic asthma compared to individuals with non-atopic asthma, or the association of TGF-β with sensitization to house dust mites, it seems interesting to extend our studies to assess TGF-β at different time points or with allergen provocation tests. Papers of some researchers suggesting correlations of higher TGF-β concentrations with a more severe course of asthma encourages further work on TGF-β, based on specific clinical parameters. Thus, it may be possible to answer the question of whether TGF-β could become a potential biomarker of airway remodeling in asthma.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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