Nasal allergen and methacholine provocation tests influence co-expression patterns of TGF-β/SMAD and MAPK signaling pathway genes in patients with asthma

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Abstract. Asthma is characterized by chronic bronchial inflammation and is a highly heterogeneous disease strongly influenced by both specific and non-specific exogenous factors. The present study was performed to assess the effect of nasal allergen provocation tests and methacholine provocation tests on the mRNA co-expression patterns of genes (*SMAD1/3/6/7*, *MPK1/3* and *TGFB1/3*) involved in SMAD and non-SMAD TGF-β signaling pathways in patients with asthma. Reverse transcription-quantitative PCR was performed on blood samples taken pre-provocation and 1 h post-provocation to assess gene expression changes. Of the 59 patients studied, allergen provocations were administered to 27 patients and methacholine provocations to 32 patients. Correlations between expression levels of studied genes were found to be influenced markedly by the challenge administered, challenge test result and time elapsed since challenge. Importantly, increases in expression levels for four gene pairs (*MAPK1-SMAD3, MAPK3-SMAD3, SMAD1-SMAD3* and *SMAD3-TGFB1*) were found to correlate significantly with asthma occurrence in the allergen provocation cohort, but not in the methacholine provocation cohort. The present study allows us to draw the conclusion that both intranasal allergen and bronchial methacholine challenges influence mRNA co-expression patterns of the *SMAD1/3/6/7, MPK1/3* and *TGFB1/3* genes.

Introduction

Asthma is the most frequently occurring chronic inflammatory disease of the respiratory tract, affecting an estimated 262 million individuals in 2019 (1). It is a heterogeneous disease characterized by episodes of bronchoconstriction and limitation of expiratory flow caused by bronchial hyperresponsiveness to a range of extrinsic factors (2-4). The clinical presentation of patients with asthma is varied and depends on complex underlying gene-gene and gene-environment interactions. Asthma can be divided into two primary endotypes based on whether T-helper cell type 2-driven inflammation responses are present. These two classical endotypes are type 2 (eosinophilic) asthma and non-type 2 (non-eosinophilic) asthma (5). After type 2 innate lymphoid cells (ILC2s) were discovered to release Th2 cytokines, asthma endotypes were more accurately divided into non-T2, T2-low and T2-high (6,7). Indeed, asthma endotyping is a highly complex topic, with the levels of several factors such as Th2 cytokines, Alarmins and Serum IgE, together with levels of activity and proliferation of cell types such as granulocytes and mast cells having to be taken into consideration. To complicate matters further, a number of asthmatic T-helper cell populations may transdifferentiate into other cell types, such as CD4 effector cells under the influence of environmental factors. This T-helper cell plasticity points towards the possibility of asthma being a mixture of overlapping states of inflammation, rather than a condition defined through rigid and stable endotypes (8). These endotypes can be subdivided further based on the presence of atopy, obesity, smoking and disease onset. Asthma symptoms differ in severity and occur mostly during episodes of hyperresponsiveness. Typical symptoms consist of wheezing, coughing, chest tightness and shortness of breath (9,10). The severity of asthma symptoms is assessed with use of the Asthma Control Questionnaire (ACQ) (11) or Asthma Control Test (ACT) (11), as per the current Global Strategy for Asthma Management and Prevention (GINA) (12) guidelines. Disease severity is measured after a period of treatment long enough to achieve and maintain symptom control and not when the symptoms initially arise (13,14).

Notably, regardless of what factor induces inflammation in a particular asthma phenotype (such as allergens or pollutants), asthmatic remodeling modelling is a direct effect of TGF-β overexpression (15,16).

TGF-β is a pleiotropic cytokine found in three isoforms (TGFβ-1, TGFβ-2 and TGFβ-3) in humans, involved in the regulation of cell proliferation (especially bronchial

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myocytes), epithelial-mesenchymal transition, stimulation of fibroblast to myofibroblast transformation and extracellular matrix (ECM) deposition. TGF-β stimulates the expression of a number of matrix proteins including collagens and basal membrane proteins (17). The TGF-β family of proteins is also responsible for suppressing T and B lymphocyte and NK cell activity, macrophage, fibroblast and eosinophil chemotaxis. TGF-β also inhibits expression of MHC class II antigens and synthesis of surfactant by type II pneumocytes (18). During the early stages of fibrosis, TGF-β also stimulates stromal cells to synthesize lysyl oxidase and other collagen crosslinking enzymes, leading to an increase in collagen network rigidity. In addition, the cytokine lowers matrix metalloproteinase expression levels, decreasing the rate of ECM degeneration. Cumulatively, these effects can lead to a fibrotic response and remodeling of the bronchial ECM (19,20).

TGF-β is first synthesized as a latent complex comprising latency-associated peptides, TGF-β and latent TGF-β binding proteins 1/3/4. This latent TGF-β is activated by proteins and enzymes such as integrins, thrombospondin-1, glycoprotein A repetitions predominant and several others. Upon activation, TGF-β creates homodimeric ligands and dimers linked by disulfide bonds. Activated TGF-β then interacts with one of several TGF-β receptors (TGFβRs) (21).

Receptors for TGF-β are present in all types of human cells (22). This emphasizes a universal significance of the entire TGF-β superfamily in the regulation of immune mechanisms. There are three recognized types of membrane receptors for this cytokine: TGFβRI, TGFβRII and TGFβRIII. A total of seven subgroups (Alk1-Alk7) can be distinguished within the TGFβRI receptor (activin receptor-like kinase 5, Alk5). The TGF-β family first of all binds to TGFβRII, which leads to the recruitment of TGFβRI and subsequently the formation of TGFβRI/TGFβRII dimer (23). The complex created in this way is responsible for the phosphorylation of the TGFβRI kinase domain, which in turn results in the activation of the SMAD pathway. Activation and stabilization of TGFβRI/TGFβRII are regulated through post translational modifications, such as phosphorylation, ubiquitylation, sumoylation and neddylation. The dimer of receptors I and II is also controlled by interactions with other proteins at the cell surface and in the cytoplasm. The two TGF-β receptors are highly conserved single transmembrane receptors with intracellular serine/threonine kinase domains (24,25).

Intracellular effectors of TGF-β signaling include, among others, the SMAD family of proteins and MAP kinases. These effectors are activated when TGF-β receptors bind their ligands. Upon activation, they translocate to the nucleus and act as transcription factors for more than 500 genes. TGF-β signaling can be conducted in a SMAD-dependent or SMAD-independent manner (26). In the canonical SMAD pathway of TGF-β signaling, upon binding TGF-β, ALK1 (or TGFβRII), a TGF-β receptor which includes an intracellular kinase domain recruits and phosphorylates ALK5 (or TGFβRI), with which it creates an activated heteromeric complex (27). The complex then phosphorylates SMAD1 (bound to SMAD5 in the SMAD1/5 dimer) which leads to the formation and activation of the trimeric SMAD1/4/5 complex. This complex then translocates into the nucleus and creates aggregates which act as a transcription factor, affecting target gene expression (28). SMAD6 and SMAD7, on the other hand, inhibit the TGF-β-SMAD intracellular signaling pathway by diminishing ALK1 and ALK5 activation and signaling. ALK5 (or TGFβRI), activated upon binding TGF-β, induces intracellular activation of MAPK, TAK1, JNK, ERK1/2 and p38 leading to modulation of target gene expression without the involvement of SMAD proteins (29). The Src tyrosine kinase, phosphatidylinositol 3'-kinase and Rho GTPases are also involved in SMAD-independent TGF-β signal transduction. The conditions for the activation of ALK1 and ALK5 differ and depend largely on the specific and non-specific activation inducing factors (30-32). Due to the nature of TGF-β/SMAD and TGF-β/MAPK signaling, the study of cellular responses to the nasal allergen challenge and methacholine challenge tests may provide a deeper understanding of asthma pathogenesis. There is a steadily increasing amount of evidence which shows that the varied responses to TGF-β signaling stem from regulatory crosstalk between the numerous pathways in which TGF-β is a component. A number of the pathways involved share receptors, transcription factors and ligands, while having diverse effects on target gene transcription (21).

Hyperactivity of TGF-β-SMAD signaling is the basis of several disorders, including organ fibrosis and progressive cancers. Elevated TGF-β expression has been found in patients with asthma, COPD, idiopathic pulmonary fibrosis, renal failure, heart failure, myocardial infarction, cystic fibrosis and systemic scleroderma (33-35). TGF-β-SMAD pathway hyperactivity leads to the development of chronic inflammation and increased myofibroblast activation. Excessive ECM deposition due to a dysregulation of TGF-β signaling is a key component of organ fibrosis and tumor formation. In the presence of certain mutations, especially those in TP53, BRAF and SMAD4, this ECM buildup can contribute to cancer-associated fibroblast formation, which ultimately leads to fibrotic and neoplastic disease (36). Therefore, TGFβRI inhibition may be a valuable strategy for the treatment of several fibrosis-related disorders (37-41).

The aim of the present study was to evaluate the effect of nasal allergen challenge and methacholine challenge test on changes in mRNA expression of genes encoding the TGF-β (*TGFB1* and *TGFB3*)-SMAD (*MAPK1/3*, *SMAD1/3/6/7*) signaling pathway in peripheral blood mononuclear cell of patients with asthma.

Materials and methods

Study groups. A sample of 71 adult patients (28 males and 43 females), including both patients with asthma and non-asthmatic patients (healthy controls) was recruited from several departments of the N. Barlicki University Clinical Hospital No. 1 of the Medical University of Lodz. In particular, the Department of Pulmonology and Allergology, the Department of General and Oncological Pulmonology and the Department of Internal Medicine, Asthma and Allergy. Additionally, patients were recruited from the Specialist Outpatient Clinic of Pulmonary Diseases and Allergology of the same hospital. Patient age ranged from 19-71 years. Patient recruitment was conducted between March 2014 and February 2017. All patients enrolled in the study were subjected to standard clinical practice. All patients volunteered to take part in the

study. Survey questionnaire data was collected with help from medical doctors specialized in the relevant fields (allergology, pulmonology and internal medicine). The data were collected on the basis of individual patient medical documentation from inpatient and outpatient treatment. As a number of patients were receiving other treatments, washout periods were considered and put in place before carrying out provocations and collecting blood samples. Upon being qualified for the study, the patients were administered either a methacholine test or an intranasal allergen challenge, in full accordance with relevant medical recommendations. Next, peripheral venous blood samples were collected from the patients, before the challenge (coded 0 h) and again, 1 h after the challenge (coded 1 h). All blood samples were taken from the ulnar vein. Patients with asthma were included in the study based on having received a code J45 ICD-10 classification. The patient population was divided into two cohorts, depending on which challenge (allergen or methacholine) they received. There were no participants who would have received both challenges at the same time. The present study was conducted in 2019-2020 and was approved by the local Ethics Committee (the Research Review Board of the Medical University of Lodz, Lodz, Poland; approval no. RNN/31/14/KE). At the beginning of the present study, the participants were invited to participate voluntarily and a written informed consent was obtained from every patient prior to enrolment.

Asthma diagnosis. The global initiative for asthma (GINA) 2019 recommendations (12), were used to identify patients with asthma, based on lung function test results and the presence of clinical asthma symptoms. GINA Report Guidelines were also used to evaluate asthma control and severity levels. Medical history data was taken from medical records of patients. If a particular patient had not had allergy tests and spirometry performed in the past, those tests were performed as part of the study recruitment visit. Patient exclusion criteria for the study included: Signs of viral infections (both generalized and respiratory), presence of clinically significant asthma exacerbations, ongoing therapy with pharmaceuticals such as rifampicin or phenobarbital, which may induce glucocorticoid resistance, and failure to comply with recommendations from the supervising medical professional. The control group in the present study consisted of healthy, patients without asthma. Inclusion criteria for this group were as following: No allergy symptoms or history, no atopic dermatitis symptoms or history, no aspirin hypersensitivity signs or history, no bronchial asthma signs or history, no signs or history of other pulmonary diseases, negative allergen skin prick test results, no bronchial asthma or atopic disorders in first degree relatives. Spirometry was performed in full accordance with the European Respiratory Society (ERS)/American Thoracic Society (ATS) standards (42). Allergological testing was performed in full accordance with the European Academy of Allergy and Clinical Immunology (EAACI) guidelines (43). Patients were chosen to be administered either a nasal allergen challenge or a methacholine challenge based on clinical indications. Patients with diagnosed atopy were administered allergen challenges, while non-atopic patients were administered methacholine challenges.

Nasal allergen challenges (NAC). NAC were also performed in accordance with current EAACI standards (43) and procedures and recommendations provided by the test manufacturer (Allergopharma challenge test solutions; Allergopharma GmbH & Co. KG). The NAC tests were performed in accordance with the No. 9531 protocol, available from the manufacturer's website (https://www.allergopharma.com/home/). The allergens used were chosen in accordance with patient allergen sensitivity, based on individual clinical data.

Methacholine challenge test. The challenge was performed according to the *ERS technical standard on bronchial challenge testing: general considerations and performance of methacholine challenge tests* (42).

Expression of mRNA by reverse transcription-quantitative (RT-q)PCR. Patient blood samples were stored in hematology tubes containing tripotassium ethylenediaminetetraacetic acid (EDTA-K3; Sartorius AG). Patient genetic material was isolated from the peripheral blood leukocyte fraction using the QIAamp DNA Blood Mini Kit (Qiagen GmbH) according to the manufacturer's protocols. Approximately 500,000 cells per sample were used for RNA extraction. RNA extraction was performed according to the standard acid-guanidinium-phenol-chloroform method, with use of the TRI Reagent Solution (Ambion; Thermo Fisher Scientific, Inc.). RNA samples were analyzed via gel electrophoresis. Only samples with well-preserved 28S, 18S and 5S rRNA bands were used for further analysis. RNA concentration in the purified samples was measured via spectrophotometry at wavelengths of 260 and 280 nm (ND-100; Nanodrop Technologies; Thermo Fisher Scientific, Inc.). Samples which exhibited a ratio of 260 and 280 nm measurements between 1.8 and 2.1 were determined to be sufficiently pure for further analysis. Next, RT-qPCR was performed to analyze expression levels of the examined genes. These analyses were performed in the Laboratory of Personalized Medicine and Biotechnology of the BioNanoPark, Regional Science and Technology Park in Lodz (Lodz, Poland). The expression levels of 8 genes were studied, aside from the internal control: *MAPK1* and *MAPK3, SMAD1, SMAD3, SMAD6* and *SMAD7* and *TGFB1* and *TGFB3*. β-2M exhibited a constant expression level in the tested samples. β-2M was chosen as the reference gene and used as internal control for normalization in all RT-qPCR reactions, as all other potential reference genes exhibited suboptimal stability. Commercially available TaqMan probes (Thermo Fisher Scientific, Inc.) (hybridization probes used to increase qPCR reaction specificity) for the eight studied genes and internal control were chosen. None of the chosen probes react with genomic DNA. Table I contains assay ID codes for the probes used. All probes were tagged with the FAM fluorophore by the manufacturer. The primers' manufacturer (Thermo Fisher Scientific, Inc.) did not publish the primer sequences for the probes used in this study, only amplicon data. All primer data are publicly available from the manufacturer's website (https://www.thermofisher.com/taqman-gene-expression/product/Hs00195432_m1, etc.). The TaqMan probes and Master Mixes used are both commercially available (Biotium, Inc.). RT-qPCR assays were performed with use of a Real-Time PCR Optical Thermocycler (Biometra Biomedizinische

Table I. Analyzed genes and the applied TaqMan probes.

Gene	Assay ID
SMAD1	Hs00195432_m1
SMAD3	Hs00969210_m1
SMAD6	Hs00178579_m1
SMAD7	Hs00998193_m1
TGF-β1	Hs00998133_m1
TGF- β3	Hs01086000_m1
MAPK1	Hs01046830_m1
MAPK3	Hs00385075_m1
β-2M	Hs00187842_m1

Table II. Reverse transcription-quantitative PCR conditions for the analyzed expression of the studied genes.

Stage	Temperature, ˚C	Time, sec	Number of cycles
UNG incubation	50	120	1
Polymerase activation	95	600	1
Denaturation	95	15	45
Annealing	60	60	45

UNG, Uracil-DNA glycosylase.

Analytik GmbH). The PCR experiments consisted of two stages: Reaction condition optimization stage and evaluation of expression levels for each studied gene for each patient sample. Table II presents details of the RT-qPCR reaction conditions. PCR was performed in two repetitions for each gene and each patient sample. Quantification cycle (Cq) values averaged for the two replicates are the primary body of results for the present study. Cq values are defined as the number of PCR cycles required for the fluorescent signal to cross the threshold of visibility. It is possible to estimate the amount of pre-reaction cDNA amount in a given sample based on the Cq value, which allows for gene expression analysis. The MX-Pro software (ver. 3.2; Stratagene; Agilent Streck LLC) was used to calculate Cq values for each sample. Cq values of each studied gene were compared with the internal control values to determine ΔCq values. The MX-Pro data analysis module was used to automatically calculate PCR data and analyze results according to the $2^{-\Delta\Delta Cq}$ method (44). A reaction yield of 100% was assumed. Standard curves were constructed to validate PCR efficiency.

Statistical analysis. Statistical analysis was conducted using Statistica 13.1 (TIBCO) software. χ^2 (or Fisher's, where appropriate) tests were used to compare nominal values. Comparisons of linear data were performed using Spearman's correlation; to avoid incorrect interpretation of the results (e.g. false correlation resulted from outliers) it was decided to publish all data distribution charts in the supplementary material. All measured results were considered significant at α level=0.05. The α level is the threshold value against which P-values are measured. P<0.05 was considered to indicate a statistically significant difference.

Results

In total, 71 patients were recruited as study participants. Of those 71, 10 (14%) patients were excluded from the final results due to qPCR non-detects for some, or all of the studied genes. Additionally, 2 (3%) more patients were excluded due to the lack of methacholine challenge test results. Of the 59 patients included in the final study, 37 were non-asthmatic subjects and 22 were patients with asthma. Table III presents detailed characteristics of the study populations, including demographic and clinical parameters and P-values for comparisons of subjects with and without asthma, calculated via the two-sided χ^2 test, or Fisher's exact test, where appropriate. Of the 59 patients, 32 (54%) were administered a methacholine challenge and 27 (46%) were administered an intranasal allergen challenge. All patients who exhibited a positive methacholine provocation test result received an asthma diagnosis, including patients not previously diagnosed with asthma.

Mean Cq values for the RT-qPCR protocols ranged from 32,023 for *TGFB1* at the lowest to 38,861 for *SMAD6* at the highest. For all studied genes, a varying proportion of samples gave non-detect qPCR results. TGFB1 had the fewest at 2,75% and SMAD6 the most, at 84,6%. A summary of qPCR data for all studied genes can be found in Table IV.

Correlation assays were performed for the expression levels of every possible pairing of the studied genes. Separate assays were performed for base (pre-challenge) samples and the samples taken 1 h post-challenge. Correlation levels were found to be significantly influenced by the challenge administered, challenge test result and time elapsed since challenge.

The co-expression patterns for the methacholine negative, methacholine positive and allergen negative pre-challenge groups exhibited few statistically significant differences. Expression levels of most gene pairings were found to be strongly correlated in these populations. Only *MAPK1* and *TGFB1* paired with other studied genes (*MAPK3*, *SMAD1*, *SMAD3*, *SMAD6*, *SMAD7* and *TGFB3*) significantly differed in correlation values between these populations.

The methacholine positive post-challenge group however, exhibited numerous significant differences in co-expression patterns, when compared with the methacholine negative, methacholine positive and allergen negative pre-challenge groups. In particular, only the changes in expression levels for *MAPK1-MAPK3*, *MAPK1-TGFB1*, *MAPK3-SMAD6*, *SMAD1-SMAD3*, *SMAD1-TGFB3*, *SMAD3-TGFB1/3* and *SMAD6-SMAD7* were found to be significantly correlated.

By contrast, pre-challenge expression levels for most gene pairs in the allergen positive group were not significantly correlated. The only statistically significant correlations in this group were found for *MAPK1-MAPK3*, *SMAD3-MAPK1/3*, *SMAD1-SMAD6/7* and *TGFB3-SMAD3/6/7*. The post-challenge results for this population also presented several co-expressing gene pairs. In particular, the changes in expression levels for *MAPK3-SMAD1*, *MAPK3-TGFB3*, *SMAD1-SMAD3/6*, *SMAD1-TGFB1*, *SMAD3-SMAD6*, *SMAD3-TGFB1* and *SMAD6-SMAD7* were found to be

Table III. Demographic and clinical characteristics of study participant population divided into subjects with asthma and subjects without asthma.

Variable	Value		P-value for comparison of subjects with vs. without asthma
	Subjects without asthma (n=37)	Subjects with asthma (n=22)	
Sex			0.910
Male	14 (37.8)	8 (36.4)	
Female	23 (62.2)	14 (63.6)	
Age, years	37.3±14.4; 33.0 (25.0-48.0)	38.4±14.7; 32.0 (28.0-54.0)	0.610
BMI, kg/m^2	25.2±5.4, 25.2 (21.2-28.7)	25.2±4.2; 24.5 (21.5-29.3)	0.748
Allergy			
Seasonal	15 (40.5)	9 (40.9)	0.978
Perennial	12 (32.4)	9 (42.9)	0.427
Number of allergens	0 (1-4); full range, 0-7	1 (0-4); full range, 0-8	0.517
Smoking			
Current smokers[a]	7 (18.9)	6 (27.3)	0.524
Ex-smokers[a]	10 (27.0)	5 (22.7)	0.767
Number of pack years	0 (0-6); full range, 0-24	0.5 (0-3); full range, 0-30	0.875
Rhinitis			
Rhinitis[a]	26 (70.3)	20 (90.9)	0.104
nGC rhinitis treatment	10 (27.0)	6 (27.3)	0.983
Episodic rhinitis[a]	7 (18.9)	7 (31.8)	0.345
Chronic rhinitis	21 (56.8)	13 (59.1)	0.860
Seasonal rhinitis[a]	9 (24.3)	7 (31.8)	0.558
Perennial rhinitis	19 (51.4)	13 (59.1)	0.723
Medications			
Anti-H1 treatment	12 (32.4)	11 (50.0)	0.181
Proton pump inhibitor treatment[a]	3 (8.1)	2 (9.1)	1.000
Medications hypersensitivity[a]	2 (5.4)	1 (4.6)	1.000
Comorbidities			
Nasal polyps[a]	2 (5.4)	0 (0.0)	1.000
Lipid disorders[a]	2 (5.4)	2 (9.1)	0.624
Hypothyroidism	0	0	-
Thyroid goiter	0	0	-
Hyperthyroidism[a]	3 (8.1)	1 (4.6)	1.000
Atherosclerosis	0	0	-
Hypertension[a]	5 (13.5)	2 (9.1)	0.702
Arrhythmiav[a]	2 (5.4)	0 (0.0)	0.524
Coronary heart disease	0	0	-
Myocardial infarction	0	0	-
Other cardio-vascular diseases	0	0	-
Chronic obstructive pulmonary disease	0	0	-
Other pulmonary diseases[a]	0	1 (4.6)	0.373
Peptic ulcer disease[a]	2 (5.4)	1 (4.6)	1.000
Duodenal ulcer disease	0	0	-
Neoplastic diseases	0	0	-
Immunodeficiency[a]	1 (2.7)	1 (4.6)	1.000

Values are expressed as n, n (%), the mean ± standard deviation and/or median (interquartile range) unless otherwise indicated. P-values were calculated using the two-sided χ^2 test or [a]Fisher's exact test. IQR, interquartile range; NGC, nasal glucocorticosteroid.

Table IV. Summary of quantitative PCR values for the studied genes.

Gene	Not-detected, %	Mean Cq†
MAPK1	7.82	36.106
MAPK3	8.78	36.894
SMAD1	30.58	37.486
SMAD3	5.55	35.284
SMAD6	84.60	38.861
SMAD7	27.65	37.327
TGFB1	2.75	32.023
TGFB3	68.04	37.694

Cq, quantification cycle

significantly correlated. Tables V-X presented the statistical data for these analyses.

Additionally, two-sided χ^2 tests with Fisher's correction were performed to study the correlation between bronchial asthma occurrence and co-expression of each gene pairing. No significant correlations were found in the methacholine challenge group. Increased expression levels for four gene pairs in the allergen provocation group were found to significantly correlate with occurrence of bronchial asthma. Namely, *MAPK1-SMAD3* [P=0.002; odds ratio (OR)=42.780], *MAPK3-SMAD3* (P=0.010; OR=24.000), *SMAD1-SMAD3* (P=0.034; OR=14.250) and *SMAD3-TGFB1* (P=0.020; OR=17.000). The statistical data related to these analyses are presented in Table XI.

In addition, Spearman's rank correlation coefficient tests were performed to analyze the correlation between patient age, body mass index and number of pack years and expression levels of each of the studied genes. No significant correlations were discovered.

Figs. S1-18 provide additional graphical depictions of the correlations described in this section.

Discussion

The present study revealed several differences between the effects of non-specific (methacholine) and specific (allergen) challenges on the levels of mRNA expression of the main genes of the TGF-β signaling pathways and the correlations between these changes, especially in subjects with asthma. Notably, the present study did not consider the absolute values of gene expression, only the degrees of correlation between the expression levels for the studied genes. A previous study analyzed the changes in absolute values of expression for these genes, in similar conditions (45). The present study was an original project, not directly based on any previously published studies. There are currently no directly analogous, publicly available studies on the influence of asthma and provocation tests on the expression patterns of genes. However, there are a number of studies indirectly related to the subject of the present study, a number of which are discussed in the following section, such as Yu *et al* (46), Goumans *et al* (47) and Fredriksson *et al* (48).

Regarding the methacholine challenge results, in the pre-challenge sample group, methacholine challenge results of patients (positive or negative) were not found to significantly influence co-expression patterns of the studied gene pairings. The methacholine bronchoprovocation test is highly sensitive when used for the detection and quantification of airway hyperresponsiveness (AHR) (49,50). The test can be used to exclude clinically significant AHR if the concentration of methacholine required to reduce a subject's forced expiratory volume in 1 sec by 20% is >16 mg/ml (51). As AHR severity is correlated with clinical severity of asthma phenotypes, AHR measured via the methacholine test can be used as a diagnostic tool for asthma (52,53). However, while the test exhibits excellent sensitivity when used to detect AHR in clinically significant asthma, it is characterized by poor specificity. A number of other diseases, including chronic obstructive pulmonary disease, cystic fibrosis and allergic rhinitis may also cause AHR. As such, a positive methacholine challenge test has to be interpreted carefully and does not suffice as a standalone diagnostic tool for asthma (54,55).

This is reflected in the pre-challenge results for both the positive and negative result cohorts, which did not exhibit statistically significant differences in gene co-expression patterns. Despite the patients exhibiting clinically significant AHR after the challenge, at the genetic level the pre-challenge correlation levels for the positive result cohort were similar to those of the negative result cohort. This might signify that the TGF-β signaling pathways were functioning normally and no hyperactivity was present pre-provocation, or the particular asthma endotype did not significantly influence gene expression of the studied genes (56). Last, the analysis of correlation levels between the occurrence of asthma and gene co-expression for the post-challenge samples in the positive methacholine challenge group did not yield any statistically significant results. Notably, this does not necessarily signify a lack of pathway activation as understood by an increase in gene expression. The lack of co-expression or concurrent activity increases across the TGF-β signaling pathway in patients with asthma might point to a different inflammatory pathway such as the Toll-like receptor or NF-κB pathway having been triggered by the methacholine provocation instead (57). Another possibility is that some of the genes in the TGF-β were activated, but not co-expressed with the rest of the studied proteins, or that the changes were too slight to be detected.

Regarding the nasal allergen challenge (NAC) results, for both pre-provocation, and post-provocation samples, the NAC test is characterized by higher specificity compared with the methacholine test. This fact is reflected at the genetic level by the strong differences in gene expression levels between the negative antigen challenge result and positive antigen challenge result populations. However, care needs to be taken when interpreting positive NAC results, as allergic rhinitis or other atopic conditions can also cause a reaction to the allergen challenge (58-61). The pre-challenge correlation levels for the negative result cohort of the allergen challenge were similar to those of the pre-challenge results for the methacholine challenge population. However, pre-challenge correlation levels for the positive test result cohort differed significantly, with far fewer gene pairs exhibiting strong correlations. This difference between cohorts may reflect the high specificity of the NAC

Table V. Correlations of base expression (pre-challenge) levels within pairs of studied genes in patients who exhibited a negative methacholine challenge test result.

Methacholine negative-0 h	*MAPK1-A*	*MAPK3-A*	*SMAD1-A*	*SMAD3-A*	*SMAD6-A*	*SMAD7-A*	*TGFB1-A*	*TGFB3-A*
MAPK1-A	-	0.68; 0.002	0.14; 0.567	0.12; 0.642	-0.02; 0.942	0.28; 0.256	-0.02; 0.938	0.04; 0.871
MAPK3-A	0.68; 0 002	-	0.63; 0.005	0.58 0.012	0.51; 0.030	0.45; 0.061	0.26; 0.303	0.51; 0.031
SMAD1-A	0.14; 0.567	0.63; 0.005	-	0.76; <0.001	0.80; <0.001	0.59; 0.010	0.26; 0.010	0.69; 0.002
SMAD3-A	0.12; 0.642	0.58; 0.012	0.76; <0.001	-	0.94; <0.001	0.71; 0.001	0.61; 0.008	0.76; <0.001
SMAD6-A	-0.02; 0.942	0.51; 0.030	0.80; <0.001	0.94; <0.001	-	0.72; 0.001	0.41; 0.090	0.88; <0.001
SMAD7-A	0.28; 0.256	0.45; 0.061	0.59; 0.010	0.71; 0.001	0.72; 0.001	-	0.36; 0.139	0.79; <0.001
TGFB1-A	-0.02; 0.938	0.26; 0.303	0.26; 0.010	0.61; 0.008	0.41; 0.090	0.36; 0.139	-	0.35; 0.160
TGFB3-A	0.04; 0.871	0.51; 0.031	0.69; 0.002	0.76; <0.001	0.88; <0.001	0.79; <0.001	0.35; 0.160	-

Top values in each cell represent the Spearman's rank correlation coefficient, while bottom values represent the P-value for each pair of studied genes.

Table VI. Correlations of base (pre-challenge) expression levels within pairs of studied genes in patients who exhibited a positive methacholine challenge test result.

Methacholine positive-0 h	*MAPK1-A*	*MAPK3-A*	*SMAD1-A*	*SMAD3-A*	*SMAD6-A*	*SMAD7-A*	*TGFB1-A*	*TGFB3-A*
MAPK1-A	-	0.47; 0.077	0.41; 0.131	0.47; 0.077	0.40; 0.143	0.53; 0.043	-0.36; 0.193	0.43; 0.107
MAPK3-A	0.47; 0.077	-	0.87; <0.001	0.86; <0.001	0.89; <0.001	0.86; <0.001	0.20; 0.475	0.91; <0.001
SMAD1-A	0.41; 0.131	0.87; <0.001	-	0.85; <0.001	0.87; <0.001	0.87; <0.001	0.38; 0.166	0.91; <0.001
SMAD3-A	0.47; 0.077	0.86; <0.001	0.85; <0.001	-	0.73; 0.002	0.80; <0.001	0.16; 0.558	0.81; <0.001
SMAD6-A	0.40; 0.143	0.89; <0.001	0.87; <0.001	0.73; 0.002	-	0.76; 0.001	0.30; 0.277	0.93; <0.001
SMAD7-A	0.53; 0.043	0.86; <0.001	0.87; <0.001	0.80; <0.001	0.76; 0.001	-	0.24; 0.398	0.79; <0.001
TGFB1-A	-0.36; 0.193	0.20; 0.475	0.38; 0.166	0.16; 0.558	0.30; 0.277	0.24; 0.398	-	0.27; 0.328
TGFB3-A	0.43; 0.107	0.91; <0.001	0.38; 0.166	0.81; <0.001	0.93; <0.001	0.79; <0.001	0.27; 0.328	-

Top values in each cell represent the Spearman's rank correlation coefficient, while bottom values represent the P-value for each pair of studied genes. Statistically significant results presented in bold font.

test. Positive NAC results indicate the presence of an allergic reaction. Chronic allergy-related inflammatory responses in these patients may have led to established, constant changes in the expression levels of TGF-β signaling pathway genes, which could be detectable via qPCR even in samples taken prior to allergen provocation (62-64).

Table VII. Correlations of the change in expression levels (1 h post-challenge) within pairs of studied genes in patients who exhibited a positive methacholine challenge test result.

Methacholine positive-1 h	*MAPK1-B-A*	*MAPK3-B-A*	*SMAD1-B-A*	*SMAD3-B-A*	*SMAD6-B-A*	*SMAD7-B-A*	*TGFB1-B-A*	*TGFB3-B-A*
MAPK1-B-A	-	0.38; 0.164	0.22; 0.435	0.20; 0.475	0.19; 0.499	0.11; 0.704	-0.02; 0.940	0.40; 0.136
MAPK3-B-A	0.38; 0.164	-	0.58; 0.025	0.39; 0.147	0.50; 0.056	0.50; 0.058	0.12; 0.666	0.65; 0.008
SMAD1-B-A	0.22; 0.435	0.58; 0.025	-	0.61; 0.016	0.92; <0.001	0.45; 0.089	0.56; 0.030	0.44; 0.101
SMAD3-B-A	0.20; 0.475	0.39; 0.147	0.61; 0.016	-	0.61; 0.016	-0.09; 0.742	0.69; 0.005	0.30; 0.283
SMAD6-B-A	0.19; 0.499	0.50; 0.056	0.92; <0.001	0.61; 0.016	-	0.53; 0.043	0.51; 0.050	0.42; 0.121
SMAD7-B-A	0.11; 0.704	0.50; 0.058	0.45; 0.089	-0.09; 0.742	0.53; 0.043	-	-0.10; 0.732	0.45; 0.089
TGFB1-B-A	-0.02; 0.940	0.12; 0.666	0.56; 0.030	0.69; 0.005	0.51; 0.050	0.10; -0.732	-	0.26; 0.355
TGFB3-B-A	0.40; 0.136	0.65; 0.008	0.44; 0.101	0.30; 0.283	0.42; 0.121	0.45; 0.089	0.26; 0.355	-

Top values in each cell represent the Spearman's rank correlation coefficient. while bottom values represent the P-value for each pair of studied genes. Statistically significant results presented in bold font.

Table VIII. Correlations of base (pre-challenge) expression levels within pairs of studied genes in patients who exhibited a negative allergen challenge test result.

Allergen negative-0 h	*MAPK1-A*	*MAPK3-A*	*SMAD1-A*	*SMAD3-A*	*SMAD6-A*	*SMAD7-A*	*TGFB1-A*	*TGFB3-A*
MAPK1-A	-	0.21; 0.610	0.29; 0.493	0.19; 0.649	0.19; 0.651	0.31; 0.456	0.40; 0.320	0.29; 0.490
MAPK3-A	0.21; 0.610	-	0.93; 0.001	0.80; 0.017	0.76; 0.028	0.93; 0.001	0.81; 0.015	0.79; 0.020
SMAD1-A	0.29; 0.493	0.93; 0.001	-	0.72; 0.045	0.90; 0.002	0.93; 0.001	0.93; 0.001	0.86; 0.006
SMAD3-A	0.19; 0.649	0.80; 0.017	0.72; 0.045	-	0.75; 0.031	0.75; 0.031	0.79; 0.020	0.77; 0.025
SMAD6-A	0.19; 0.651	0.76; 0.028	0.90; 0.002	0.75; 0.031	-	0.83; 0.010	0.93; 0.001	0.90; 0.002
SMAD7-A	0.31; 0.456	0.93; 0.001	0.93; 0.001	0.75; 0.031	0.83; 0.010	-	0.86; 0.007	0.93; 0.001
TGFB1-A	0.40; 0.320	0.81; 0.015	0.93; 0.001	0.79; 0.020	0.93; 0.001	0.86; 0.007	-	0.90; 0.002
TGFB3-A	0.29; 0.490	0.79; 0.020	0.86; 0.006	0.77; 0.025	0.90; 0.002	0.93; 0.001	0.90; 0.002	-

Top values in each cell represent the Spearman's rank correlation coefficient. while bottom values represent the P-value for each pair of studied genes. Statistically significant results presented in bold font.

The expression levels of several gene pairs have also been shown to change with strong correlation in the samples collected 1 h post-provocation from the positive allergen provocation patient cohort. Unsurprisingly, the expression levels of *TGFB1* were correlated with *MAPK1* and *MAPK3*. TGF-β1 phosphorylates MAP kinases, including

Table IX. Correlations of base (pre-challenge) expression levels within pairs of studied genes in patients who exhibited a positive allergen challenge test result.

Allergen positive-0 h	*MAPK1-A*	*MAPK3-A*	*SMAD1-A*	*SMAD3-A*	*SMAD6-A*	*SMAD7-A*	*TGFB1-A*	*TGFB3-A*
MAPK1-A	-	0.76; <0.001	0.29; 0.240	0.62; 0.006	-0.09; 0.723	0.13; 0.606	0.06; 0.807	0.04; 0.880
MAPK3-A	0.76; <0.001	-	0.33; 0.188	0.77; <0.001	0.25; 0.320	0.17; 0.516	0.07; 0.779	0.31; 0.206
SMAD1-A	0.29; 0.240	0.33; 0.188	-	0.42; 0.081	0.56; 0.015	0.54; 0.024	0.15; 0.542	0.40; 0.101
SMAD3-A	0.62; 0.006	0.77; <0.001	0.42; 0.081	-	0.31; 0.203	0.37; 0.149	0.12; 0.648	0.56; 0.015
SMAD6-A	-0.09; 0.723	0.25; 0.320	0.56; 0.015	0.31; 0.203	-	0.70; 0.002	0.37; 0.125	0.82; <0.001
SMAD7-A	0.13; 0.606	0.17; 0.516	0.54; 0.024	0.37; 0.149	0.70; 0.002	-	0.17; 0.507	0.65; 0.005
TGFB1-A	0.06; 0.807	0.07; 0.779	0.15; 0.542	0.12; 0.648	0.37; 0.125	0.17; 0.507	-	0.34; 0.161
TGFB3-A	0.04; 0.880	0.31; 0.206	0.40; 0.101	0.56; 0.015	0.82; <0.001	0.65; 0.005	0.34; 0.161	-

Top values in each cell represent the Spearman's rank correlation coefficient, while bottom values represent the P-value for each pair of studied genes. Statistically significant results presented in bold font.

isoforms 1 and 3 (*MAPK1*, *MAPK3*), leading to the activation of SMAD-independent signaling pathways, ultimately resulting in the inhibition of MMP gene expression and inhibition of MHC class II antigen expression by pneumocytes. These effects are considered to induce asthmatic bronchial remodeling (65,66).

Allergen exposure has been shown to activate the intracellular MAPK/ERK and p38 MAPK pathways and induce IL-25 and TSLP expression *in vitro* and *in vivo* (46). However, there is a lack of published data on the influence of allergens on expression levels and co-expression patterns of the particular genes of these pathways.

The expression levels of *SMAD3* and *TGFB1* were shown to be strongly correlated following allergen provocation. Upon TβRI activation, *SMAD3* forms a dimer with SMAD 2 and takes part in TGF-β pathway signal transduction, ultimately activating nuclear transcription factors and influencing the expression of target genes such as those of MMPs, *PAI-1*, *CTGF*, *MCP-1*, *IL-6*, *TGF-β*, *TSP-1*, *TGFR-1/2*, fibronectin and proteoglycans, as well as type I and III collagen (46,67). This mechanistic relation can explain the strong correlation between *SMAD3* and *TGFB1* levels. As levels of TGF-β increase as part of the inflammatory response, more TβRI is activated and leads to expression and recruitment of more SMAD3 proteins.

Frequently, in pulmonary allergic reactions, and especially in asthma, TGF-β activity is greatly increased. This hyperactivity leads to the recruitment of numerous leukocytes (mainly macrophages and granulocytes) (67) to the pulmonary tissue through chemotaxis, which is a critical element in any maintained inflammatory reaction. The fibrogenic and immunomodulatory activities of TGF-β also play significant roles in asthma, leading to asthmatic airway remodeling (68). In addition, TGF-β1 also induces differentiation of TH17 lymphocytes. These cells are able to produce high amounts of IL-17, which further maintains acute inflammation in the pulmonary tissue (69).

TGF-β3 is known to be a 'switch', which shifts signaling activity away from the TGF-β1/SMAD2/3 signaling axis and potentiates the signaling performed by Alk1/SMAD1 in lung fibroblasts. In endothelial tissues, the balance between these two signaling pathways has been shown to exert strong effects on vascular homeostasis (47,70). The TGF-β1/SMAD2/3 pathway is shown to inhibit endothelial cell migration and proliferation, while the Alk1/SMAD1 pathway is shown to induce endothelial cell migration and proliferation (71). The effects of this balance and disruptions thereof are a topic which is not well understood in the context of asthma and would greatly benefit from further research.

In the present study, *TGFB3* expression was found to correlate strongly with *SMAD1* expression, which is consistent with previously described mechanisms. Notably, *SMAD3* expression was also found to strongly correlate with *TGFB3* and *SMAD1* expression. Perhaps this co-expression might stem from a shift from *SMAD2/3* to *SMAD1/5/8* signaling, mediated by an increase in TGF-β3 levels in response to the raise in *SMAD3* activity due to the allergic reaction (72) potentially constituting a novel interaction within this signaling pathway.

SMAD6 and *SMAD7* were also shown to be co-expressed in this context. The SMAD6 and SMAD7 proteins act as a negative feedback mechanism for the SMAD-dependent TGF-β signaling pathway. These proteins form a dimer which

Table X. Correlations of the change in expression levels (1 h post-challenge) within pairs of studied genes in patients who exhibited a positive allergen challenge test result.

Allergen positive-1 h	*MAPK1-B-A*	*MAPK3-B-A*	*SMAD1-B-A*	*SMAD3-B-A*	*SMAD6-B-A*	*SMAD7-B-A*	*TGFB1-B-A*	*TGFB3-B-A*
MAPK1-B-A	-	0.74; <0.001	0.32; 0.213	0.40; 0.097	0.36; 0.137	0.28; 0.273	0.51; 0.030	0.07; 0.801
MAPK3-B-A	0.74; <0.001	-	0.39; 0.117	0.40; 0.101	0.48; 0.046	0.23; 0.384	0.35; 0.157	-0.06; 0.808
SMAD1-B-A	0.32; 0.213	0.39; 0.117	-	0.64; 0.005	0.46; 0.061	0.48; 0.054	0.18; 0.492	0.55; 0.026
SMAD3-B-A	0.40; 0.097	0.40; 0.101	0.64; 0.005	-	0.45; 0.060	0.29; 0.264	0.61; 0.007	0.67; 0.003
SMAD6-B-A	0.36; 0.137	0.48; 0.046	0.46; 0.061	0.45; 0.060	-	0.60; 0.010	0.22; 0.385	0.51; 0.038
SMAD7-B-A	0.28; 0.273	0.23; 0.384	0.48; 0.054	0.29; 0.264	0.60; 0.010	-	0.19; 0.468	0.17; 0.520
TGFB1-B-A	0.51; 0.030	0.35; 0.157	0.18; 0.492	0.61; 0.007	0.22; 0.385	0.19; 0.468	-	0.34; 0.181
TGFB3-B-A	0.07; 0.801	-0.06; 0.808	0.55; 0.026	0.67; 0.003	0.51; 0.038	0.17; 0.520	0.34; 0.181	-

Top values in each cell represent the Spearman's rank correlation coefficient, while bottom values represent the P-value for each pair of studied genes. Statistically significant results presented in bold font.

inhibits TGF-β signaling pathway activity by tagging activated TβRI for proteasomal degradation. The roles of *SMAD6* and *SMAD7* in asthma are not yet fully understood (73).

The final analysis which led to meaningful findings was the analysis of correlation between increased expression of tested gene pairs and the occurrence of bronchial asthma in allergen challenge patient cohort, 1 h post-provocation (Table XI).

A total of four gene pairs were found to be strongly correlated in the present study. First, *MAPK1* (also known as *ERK1*) and *MAPK3* (also known as *ERK2*) expression was found to correlate strongly with *SMAD3* expression. The activity of SMAD3 and both MAPK isoforms was induced by the same TGF-β receptor; Alk5. In the context of asthma, excessive TGF-β signaling by both SMAD-dependent and SMAD-independent (MAPK) pathways is well described and known to induce progression of asthmatic airway remodeling and increase the severity of asthma episodes (38). The correlation between increased expression levels for these proteins and asthma occurrence described in the present study is therefore in line with established data (30,74).

The ERK subfamily of MAPK proteins are implicated in immune cell proliferation and recruitment in asthma. The ERK1/2 signaling cascade begins with phosphorylation of the Raf-1 MAPKKK by Ras, which in turn activates MAPKK proteins (MEK1 and MEK2), which in turn, ultimately activate ERK1/2 (75). Activated ERK1/2 then phosphorylate a range of transcription factors, in particular those from the cMyc, Elk, Sap, Tal and STAT protein families (76). In asthma, the proinflammatory influence of ERK1/2 is multifactorial. Eosinophils become more susceptible to proinflammatory chemokines in the presence of IL-5. In turn, chemokines such as RANTES activate ERK1/2, which then promotes production of leukotrienes (inflammatory mediator lipids) by eosinophils, thus creating positive proinflammatory feedback (77). Eotaxin is another protein activated by ERK, which induces eosinophil recruitment and degranulation (78). Aside from its proinflammatory activity, ERK is also involved in asthmatic airway remodeling. Proinflammatory cytokines activated by ERK are implicated in epithelial barrier disruption in asthmatic airways (79). Furthermore, goblet cell hyperplasia in asthma is induced by IL-13, which is also brought about by ERK signaling (80). These findings strongly indicate the relevance of ERK signaling in the development of asthmatic symptoms. It is evident that ERK signal transduction pathways could be a valuable therapeutic target in asthma and other inflammatory conditions. Further studies on ERK function can serve to deepen the understanding of the interplay of ERK with other pathways and create foundations for pharmaceutical research.

Strong correlation was also found between the increased expression of *SMAD3* and *TGFB1* and the occurrence of asthma in the cohort examined in the present study. The underlying mechanisms of this correlation have already been explained in the previous section, which describes NAC results for pre-provocation and post-provocation samples, and the confirmation of this data by another analysis underlines its importance in the pathogenesis of asthma.

Notably, while expression levels of both MAPK isoforms were found to have a strong correlation with *SMAD3*, *MAPK1* expression was not correlated with *MAPK3* expression in this analysis, despite both the MAPK proteins being in the same signaling cascade (81).

Table XI. Correlation between increased expression within pairs of studied genes (1 h post challenge) and the occurrence of bronchial asthma-allergen challenge patient cohort.

Allergen challenge, expression level increase for gene pair	Healthy patients, n (%)	Patients with asthma, n (%)	P-value	OR	Bottom 95% CI OR	Top 95% CI OR
MAPK1-MAPK3	7 (33.3)	4 (80.0)	0.128[a]	8.00	0.75	85.73
MAPK1-SMAD1	3 (14.3)	3 (60.0)	0.062[a]	9.00	1.03	78.58
MAPK1-SMAD3	4 (19.1)	5 (100.0)	0.002[a]	42.78	1.98	926.02
MAPK1-SMAD6	4 (19.1)	3 (60.0)	0.101[a]	6.38	0.79	51.78
MAPK1-SMAD7	5 (23.8)	3 (60.0)	0.281[a]	4.80	0.62	37.35
MAPK1-TGFB1	6 (28.6)	4 (80.0)	0.055[a]	10.00	0.92	108.82
MAPK1-TGFB3	3 (14.3)	3 (60.0)	0.062[a]	9.00	1.03	78.58
MAPK3-SMAD1	4 (19.1)	2 (40.0)	0.558[a]	2.83	0.35	23.02
MAPK3-SMAD3	3 (14.3)	4 (80.0)	0.010[a]	24.00	1.95	295.08
MAPK3-SMAD6	4 (19.1)	3 (60.0)	0.101[a]	6.38	0.79	51.78
MAPK3-SMAD7	4 (19.1)	2 (40.0)	0.558[a]	2.83	0.35	23.02
MAPK3-TGFB1	7 (33.3)	3 (60.0)	0.340[a]	3.00	0.40	22.30
MAPK3-TGFB3	3 (14.3)	2 (40.0)	0.236[a]	4.00	0.46	34.92
SMAD1-SMAD3	2 (9.5)	3 (60.0)	0.034[a]	14.25	1.41	143.20
SMAD1-SMAD6	3 (14.3)	1 (20.0)	1.000[a]	1.50	0.12	18.44
SMAD1-SMAD7	2 (9.5)	2 (40.0)	0.155[a]	6.33	0.63	63.64
SMAD1-TGFB1	2 (9.5)	2 (40.0)	0.155[a]	6.33	0.63	63.64
SMAD1-TGFB3	3 (14.3)	1 (20.0)	1.000[a]	1.50	0.12	18.44
SMAD3-SMAD6	3 (14.3)	3 (60.0)	0.062[a]	9.00	1.03	78.58
SMAD3-SMAD7	3 (14.3)	3 (60.0)	0.062[a]	9.00	1.03	78.58
SMAD3-TGFB1	4 (19.1)	4 (80.0)	0.020[a]	17.00	1.47	196.44
SMAD3-TGFB3	3 (14.3)	3 (60.0)	0.062[a]	9.00	1.03	78.58
SMAD6-SMAD7	4 (19.1)	2 (40.0)	0.558[a]	2.83	0.35	23.02
SMAD6-TGFB1	4 (19.1)	2 (40.0)	0.558[a]	2.83	0.35	23.02
SMAD6-TGFB3	3 (14.3)	2 (40.0)	0.236[a]	4.00	0.46	34.92
SMAD7-TGFB1	4 (19.1)	2 (40.0)	0.558[a]	2.83	0.35	23.02
SMAD7-TGFb3	2 (9.5)	2 (40.0)	0.155[a]	6.33	0.63	63.64
TGFB1-TGFB3	4 (19.1)	3 (60.0)	0.101[a]	6.38	0.79	51.79

[a]Two-sided χ^2 test with Fisher's correction. Statistically significant results presented in bold font. OR, odds ratio; CI, confidence interval.

The last gene pair with a strong correlation in the present analysis was *SMAD1* and *SMAD3*, also described in the section which describes NAC results for pre-provocation and post-provocation samples. Increased co-expression of these genes in the context of asthma does not have a well-described mechanistic explanation. SMAD1/5/8 proteins transduce ALK 1/2/3/6 receptor signals, while SMAD 2 and 3 proteins cooperate with ALK4 and ALK5 (82,83). An analysis of ALK receptor phosphorylation patterns in response to allergen provocation might shed more light on the processes described in the present study. Perhaps, in the context of asthma, the increased co-expression of these proteins might point towards an effort to stem the hyperactivity of ALK5-mediated TGF-β signaling by increasing the activity of the antagonistic Alk1/SMAD1 pathway which could point towards a novel interaction within the TGF-β signaling pathway.

There is a large body of evidence for TGF-β having opposing roles in asthma as the disease develops. The molecule appears to have an anti-inflammatory, inhibitory effect at the onset of asthma, but switches to proinflammatory activity as the condition develops, maintaining and exacerbating pulmonary inflammation (48,84-86). This is reflected in a study on a murine model of asthma, in which rapamycin was administered in either early or established asthma (48). Rapamycin induces TGF-β activity, making the present study highly relevant to another study (84). In this study on rapamycin, airway inflammation is found to be diminished upon rapamycin administration in early asthma. In established asthma, the effect was reversed, with rapamycin having a proinflammatory effect. There is a notable lack of studies of this changing role of TGF-β in humans. The findings of the present study indicated that in patients with asthma, SMAD1 and SMAD3 were co-expressed, which might be related to this double role of TGF-β, as aforementioned. More studies are required to further study this mechanism.

In the early stages of asthma, cytokine concentrations (mainly IL-21 and IL-6) are likely below the threshold needed to induce differentiation of Th17 cells (85). Th17 lymphocyte

activity is a key inducer of neutrophilic inflammation in advanced asthma (86). It is possible that in the earlier stages of asthma, the level of Alk1/SMAD1 signaling is sufficient to counteract the pro-inflammatory activity of TGF-β and becomes insufficient as the persistent inflammation develops over the course of asthma. Thus, the antagonistic relationship between Alk1/SMAD1 and Alk5 TGF-β signaling described in the present study, might play a role in the outwardly paradoxical dual role of TGF-β throughout the different stages of asthma.

The present study was observational and patient groups were assigned based on diagnostic and therapeutic indications, but the allocation was not random. Only blood samples were collected and analyzed, therefore no comparative analysis of expression levels in different tissues could be conducted. BMP proteins which interact with Alk1/2/3/5 and can modify SMAD gene expression levels were also not taken into account in the present study. Patient blood samples were collected at only two timepoints, pre-provocation (0 h) and 1 h post-provocation. Lastly, analysis was performed only at the level of gene expression, which does not always equate the level of protein expression. A further study taking into account protein expression and analyzing a range of tissue samples would be of great benefit.

The main conclusions to be drawn from the present study are that both non-specific and specific provocations influence the co-expression patterns of a number of proteins of the TGF-β SMAD-dependent and SMAD-independent signaling pathways. The allergen challenge, but not the methacholine challenge, also influenced the co-expression patterns of some gene pairs in a manner strongly correlated with the occurrence of asthma in the patients. *TGFB1* was co-expressed with other components of the TGF-β signaling pathway, including *MAPK1*, *MAPK3* and *SMAD3*. *SMAD6* and *SMAD7*, genes of the inhibitory SMAD proteins were also found to co-express. These findings agree with the established understanding of TGF-β signaling and bronchial inflammation in asthma (30,62-64,74). The key novel finding of the present study is that two separate analyses showed *SMAD1* and *SMAD3* co-expression. The proteins encoded by these genes are understood to act in antagonistic pathways (29,82,83,87). This co-expression pattern may point towards a previously undescribed adaptive reaction to inflammation aimed to direct signaling activity away from the pro-inflammatory signaling mediated by SMAD3 and towards SMAD1 signaling. These findings serve to improve understanding of asthmatic inflammatory signaling and provide a basis for further studies in the field.

The gene co-expression patterns described in the present study, in particular the novel relationship between *SMAD1* and *SMAD3* levels, may be used to guide the development of novel therapeutics against a range of conditions besides asthma. Modern therapeutic development is frequently based on the targeting of a single cellular mechanism or protein interaction and in the future perspective, this method of pharmaceutical research will only become more dominant. At the moment, several therapeutics targeting the TGF-β/SMAD and MAPK pathways are being researched. Fresolimumab (or GC1008) is an example of such a therapeutic, an anti-TGF-β antibody developed by Genzyme which was shown to exhibit antitumor activity in renal carcinomas and melanomas with an acceptable safety profile (88). Another, developed by AstraZeneca under the name 264RAD, is an anti-αvβ6 integrin antibody shown to inhibit latent TGF-β activation. 264RAD has exhibited antitumor activity in murine models of pancreatic (89) and breast (90) cancer. PLX8394, a B-Raf inhibitor which blocks TGF-β signaling in a model of cutaneous squamous cell carcinoma has been shown to tumor cell invasion and tumor growth *in vitro* and in murine models (91).

A previously unknown interaction within the TGF-β/SMAD and MAPK cellular pathways, such as the interaction between *SMAD1* and *SMAD3* expression levels described here may prove to be a valuable and widely applicable therapeutic target, if researched further.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JP was the corresponding author and the primary writer of the manuscript. JP wrote the introduction, conducted formal analysis and was responsible for interpreting and discussing study results. AM conducted all statistical analyses, created tables and provided graphs included in supplementary data. PK was the supervisor of the present study, providing formal analysis advice and contributing to the internal reviewing and editing process. MP prepared the methodology, performed the provocations, collected blood samples and performed the RT-qPCR. JP and MP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the local Ethics Committee (consent of the Research Review Board of the Medical University of Lodz, Lodz, Poland; approval no. RNN/31/14/KE). At the beginning of the study, the participants were invited to participate in the study voluntarily and written informed consent was obtained from every patient prior to enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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