

# TGF- $\beta$ Signaling Pathways in Different Compartments of the Lower Airways of Patients With Stable COPD



Antonino Di Stefano, PhD; Claudia Sangiorgi, PhD; Isabella Gnemmi, PhD; Paolo Casolari, PhD; Paola Brun, PhD; Fabio L. M. Ricciardolo, MD; Marco Contoli, MD; Alberto Papi, MD; Pio Maniscalco, MD; Paolo Ruggeri, MD; Giuseppe Girbino, MD; Francesco Cappello, MD; Stelios Pavlides, PhD; Yike Guo, PhD; Kian Fan Chung, MD; Peter J. Barnes, MD; Ian M. Adcock, PhD; Bruno Balbi, MD; and Gaetano Caramori, MD

**BACKGROUND:** The expression and localization of transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway proteins in different compartments of the lower airways of patients with stable COPD is unclear. We aimed to determine TGF- $\beta$  pathway protein expression in patients with stable COPD.

**METHODS:** The expression and localization of TGF- $\beta$  pathway components was measured in the bronchial mucosa and peripheral lungs of patients with stable COPD (n = 44), control smokers with normal lung function (n = 24), and control nonsmoking subjects (n = 11) using immunohistochemical analysis.

**RESULTS:** TGF- $\beta$ 1, TGF- $\beta$ 3, and connective tissue growth factor expression were significantly decreased in the bronchiolar epithelium, with TGF- $\beta$ 1 also decreased in alveolar macrophages, in patients with stable COPD compared with control smokers with normal lung function. TGF- $\beta$ 3 expression was increased in the bronchial lamina propria of both control smokers with normal lung function and smokers with mild/moderate stable COPD compared with control nonsmokers and correlated significantly with pack-years of smoking. However, TGF- $\beta$ 3<sup>+</sup> cells decreased in patients with severe/very severe COPD compared with control smokers. Latent TGF- $\beta$  binding protein 1 expression was increased in the bronchial lamina propria in subjects with stable COPD of all severities compared with control smokers with normal lung function. Bone morphogenetic protein and activin membrane-bound inhibitor expression (BAMBI) in the bronchial mucosa was significantly increased in patients with stable COPD of all severities compared with control subjects. No other significant differences were observed between groups for all the other molecules studied in the bronchial mucosa and peripheral lung.

**CONCLUSIONS:** Expression of TGF- $\beta$ s and their regulatory proteins is distinct within different lower airway compartments in stable COPD. Selective reduction in TGF- $\beta$ 1 and enhanced BAMBI expression may be associated with the increase in autoimmunity in COPD.

CHEST 2018; 153(4):851-862

**KEY WORDS:** airway inflammation; autoimmunity; BAMBI; CTGF; SMAD; TGF- $\beta$

**ABBREVIATIONS:** BAMBI = bone morphogenetic proteins and activin membrane-bound inhibitor; BMP = bone morphogenetic protein; CCN2 = connective tissue growth factor; ECM = extracellular matrix; LAP = latency-associated peptide; LLC = large latent complex; LTBP = latent transforming growth factor- $\beta$  binding protein; MAPK = mitogen-activated protein kinase; PI3K = phosphoinositide 3-kinase; SMAD = small mother against decapentaplegic; TGF = transforming

growth factor; TGFBI = transforming growth factor- $\beta$ -induced protein; TGF- $\beta$ R = TGF- $\beta$  receptor; TGIF = 5'-TG-3'-interacting factor; TRAP-1 = transforming growth factor- $\beta$  receptor-associated binding protein

**AFFILIATIONS:** From the Divisione di Pneumologia e Laboratorio di Citoimmunopatologia dell'Apparato Cardio Respiratorio, Istituti Clinici Scientifici Maugeri (Drs Di Stefano, Sangiorgi, Gnemmi, and

The transforming growth factor- $\beta$  (TGF- $\beta$ ) family regulates cell proliferation, differentiation, extracellular matrix synthesis, and apoptosis, which are all important processes in COPD pathogenesis. Attenuation of TGF- $\beta$  signaling leads to pulmonary emphysema in animal models,<sup>1,2</sup> which may reflect TGF- $\beta$ 1 effects on vascular endothelial growth factor and angiogenesis.<sup>3,4</sup> TGF- $\beta$ 1 also has a pivotal role in maintaining peripheral tolerance against self-antigens<sup>5</sup> and controlling autoimmune responses.<sup>6,7</sup>

The TGF- $\beta$  superfamily has several members: TGF- $\beta$  exists as three isoforms—TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3—which exhibit similar functions *in vitro* but may have distinct activities *in vivo*.<sup>8</sup> TGF- $\beta$ s act through specific receptors (TGF- $\beta$  receptors [TGF- $\beta$ Rs]) I, II, and III. All TGF- $\beta$ s bind a heteromeric type I/II receptor complex, which activates both “canonical” signals involving SMADs (small mother against decapentaplegic) and “noncanonical” pathways involving mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K).<sup>9</sup>

---

Balbi, SpA, Società Benefit, IRCCS, Veruno (NO), Italy; the Centro Interdipartimentale per lo Studio delle Malattie Infiammatorie delle Vie Aeree e Patologie Fumo-Correlate (CEMICEF) (Drs Casolari, Contoli, Papi, and Caramori), Sezione di Medicina Interna e Cardiopulmonare, Università di Ferrara, Ferrara, Italy; the Modulo di Chirurgia Toracica (Dr Maniscalco), Azienda Ospedaliera Universitaria S. Anna, Ferrara, Italy; the Dipartimento di Medicina Molecolare (Dr Brun), Università di Padova, Padova, Italy; the Dipartimento di Scienze Cliniche e Biologiche, AOU (Dr Ricciardolo), Ospedale San Luigi, Orbassano, Università di Torino, Torino, Italy; the Unità Operativa Complessa di Pneumologia (Drs Ruggeri, Girbino, and Caramori), Dipartimento di Scienze Biomediche, Odontoiatriche e delle Immagini Morfologiche e Funzionali (BIOMORF), Università di Messina, Messina, Italy; the Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche (Dr Cappello), Sezione di Anatomia Umana, Università di Palermo, and Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy; the Department of Computing and Data Science Institute (Dr Pavlides and Guo), Imperial College London, England; the Airways Disease Section (Drs Chung, Barnes, and Adcock), National Heart and Lung Institute, Imperial College London, England; and the Priority Research Centre for Lung Health (Dr Adcock), Hunter Medical Research Institute, New Lambton Heights, NSW, Australia.

**FUNDING/SUPPORT:** This work was supported by Istituti Clinici Scientifici Maugeri, SpA, SB, IRCCS, Ricerca Corrente, FARs of the University of Ferrara (to G. C.). I. M. A. is supported by the BBSRC, the Wellcome Trust [Grant 093080/Z/10/Z], the Dunhill Medical Trust [Grant R368/0714], and the MRC-ABPI COPD-MAP consortium [Grant G1001367/1].

**CORRESPONDENCE TO:** Antonino Di Stefano, PhD, Istituti Clinici Scientifici Maugeri, IRCCS, Laboratorio di Citoimmunopatologia, dell'Apparato Cardio Respiratorio, Via per Revislate, 13, 28010 Veruno (NO), Italy; e-mail: [antonino.distefano@icsmaugeri.it](mailto:antonino.distefano@icsmaugeri.it)

Copyright © 2018 The Authors. Published by Elsevier Inc under license from the American College of Chest Physicians. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

**DOI:** <https://doi.org/10.1016/j.chest.2017.12.017>

The SMAD family of transcription factors consists of the receptor-regulated SMADs, a common pathway SMAD, and inhibitory SMADs. Receptor-regulated SMADs include SMAD2 and SMAD3, which are recognized by TGF- $\beta$ Rs and activin receptors, and SMADs 1, 5, 8, and 9, which are activated by bone morphogenetic protein (BMP) receptors. SMAD4 or cooperating SMAD is not phosphorylated by the TGF- $\beta$ Rs, whereas inhibitory SMADs (anti-SMADs), including SMAD6 and SMAD7, downregulate TGF- $\beta$  signaling. SMADs also act as signal integrators and interact with MAPK, nuclear factor- $\kappa$ B, PI3K, and hypoxia-inducible factor 1 signaling pathways.<sup>8,10</sup>

TGF- $\beta$ RIII (also known as  $\beta$ -glycan) acts as a coreceptor and directly binds TGF- $\beta$ 1, 2, and 3 to enhance their binding to the TGF- $\beta$ RI/II complex and increase SMAD-dependent signaling.<sup>11</sup> TGF- $\beta$  signaling is modulated by connective tissue growth factor (CTGF or CCN2). CNN directly binds TGF- $\beta$ 1 and facilitates binding to the TGF- $\beta$ RI/II complex enhancing downstream signaling.<sup>12</sup>

The latency-associated peptide (LAP) is associated with latent TGF- $\beta$  binding proteins (LTBPs) forming a complex known as the large latent complex (LLC).<sup>13,14</sup> Most TGF- $\beta$  is secreted as part of the LLC. LTBPs belong to the fibrillin-LTBP family of extracellular matrix (ECM) proteins. LTBP-1 anchors to the ECM and creates traction when LAP binds cell surface integrins; this traction deforms LAP, which releases active TGF- $\beta$ .<sup>13,15</sup>

Conversely, TGF- $\beta$ R-associated binding protein (TRAP) 1 inhibits TGF- $\beta$ 1 function by interfering with SMAD3 signaling.<sup>16</sup> The 5'-TG-3'-interacting factors (TGIFs), TGIF1 and TGIF2, cause repression of TGF- $\beta$ 1-activated genes by direct competition with Smad2.<sup>17</sup> In addition, BMP and activin membrane-bound inhibitor (BAMBI) acts as a competitive receptor antagonist for TGF- $\beta$ RI.<sup>18</sup> BAMBI expression is upregulated by TGF- $\beta$ 1 in a feedback loop.<sup>19</sup> TGF- $\beta$ -induced protein (TGFBI, also known as BIG-H3 and keratopithelin) is an extracellular matrix protein used as a TGF- $\beta$ 1 bioactivity marker.<sup>20</sup>

The aim of this study is to investigate the expression of TGF- $\beta$  signaling pathways in the lower airways (bronchial mucosa and peripheral lung) of patients with stable COPD and control subjects.

---

## Methods

### Subjects

All patients with COPD and healthy control subjects were recruited from the Respiratory Medicine Unit of the Istituti Clinici Scientifici

**TABLE 1 ] Clinical Characteristics of Subjects for Immunohistochemical Studies on Bronchial Biopsy Samples**

| Group                                       | No. | Age (y) | Male/Female | Pack-years of Smoking | Ex/Current Smokers | FEV <sub>1</sub> (% Predicted) Pre-β <sub>2</sub> | FEV <sub>1</sub> (% Predicted) Post-β <sub>2</sub> | FEV <sub>1</sub> /FVC (%) |
|---|-----|---------|-------------|-----------------------|--------------------|---|--|---------------------------|
| Control nonsmokers                          | 11  | 67 ± 10 | 10/1        | 0                     | 0                  | 116 ± 14  | ND   | 85 ± 10                   |
| Control smokers with normal lung function   | 12  | 61 ± 7  | 9/3         | 43 ± 26               | 2/10               | 104 ± 13  | ND   | 81 ± 6                    |
| COPD grades I and II (mild/moderate)        | 14  | 67 ± 8  | 12/2        | 40 ± 19               | 5/9                | 66 ± 14 <sup>a</sup>                              | 72 ± 12  | 60 ± 8 <sup>a</sup>       |
| COPD grades III and IV (severe/very severe) | 18  | 66 ± 9  | 11/7        | 54 ± 36               | 13/5               | 35 ± 8 <sup>a,b</sup>                             | 38 ± 9   | 44 ± 10 <sup>a,b</sup>    |

Patients with COPD were classified according to Global Initiative for Chronic Obstructive Lung Disease 2011 (<http://www.goldcopd.org>) grades of severity using only the severity of airflow obstruction. For patients with COPD, FEV<sub>1</sub>/FVC (%) are postbronchodilator values. ND = not determined; pre-β<sub>2</sub> = values obtained before bronchodilator use; post-β<sub>2</sub> = values obtained after bronchodilator use. Statistical analysis with analysis of variance test:

<sup>a</sup>P < .0001, which was significantly different from control smokers with normal lung function and control never-smokers.

<sup>b</sup>P < .0001, which was significantly different from mild/moderate COPD.

**TABLE 2 ] Characteristics of Subjects for Immunohistochemical Studies on the Peripheral Lung Tissue**

| Groups             | No. | Age (y)    | Male/Female | Ex/Current Smokers | Pack-Years of Smoking | Chronic Bronchitis | FEV <sub>1</sub> (% Predicted) | FEV <sub>1</sub> /FVC (%) |
|--------------------|-----|------------|-------------|--------------------|-----------------------|--------------------|--------------------------------|---------------------------|
| Control smokers    | 12  | 63.6 ± 3   | 10/2        | 6/6                | 51.3 ± 11.6           | No                 | 87.9 ± 4.5                     | 77.4 ± 1.7                |
| Patients with COPD | 12  | 69.9 ± 1.3 | 12/0        | 6/6                | 45.8 ± 6.1            | No                 | 68.6 ± 4.2 <sup>a</sup>        | 58.7 ± 2.5 <sup>a</sup>   |

For COPD and control smoker subjects, FEV<sub>1</sub> % predicted and FEV<sub>1</sub>/FVC % are postbronchodilator values. Data expressed as mean ± SEM.

<sup>a</sup>Analysis of variance = P < .01.

Maugeri, Veruno, Italy and the Section of Respiratory Diseases of the University Hospital of Ferrara, Italy. Archival material was used in the present study.<sup>21</sup> We obtained bronchial biopsy samples from 55 subjects to study immunohistochemically. The characteristics of these subjects are reported in Table 1. Twenty-four subjects undergoing lung resection for a solitary peripheral neoplasm were recruited for the immunohistochemical study of peripheral lung tissue. The characteristics of these subjects are reported in Table 2. COPD and chronic bronchitis were defined according to international guidelines, that is, COPD is the presence of a postbronchodilator FEV<sub>1</sub>/FVC ratio < 70% and chronic bronchitis is the presence of cough and sputum production for at least 3 months in each of two consecutive years according to Global Initiative for Chronic Obstructive Lung Disease criteria (<http://www.goldcopd.org>). In patients with COPD, the severity of the airflow obstruction was graded using the 2011 GOLD criteria. The GOLD criteria used here to stratify the severity of stable COPD was based on the degree of airflow obstruction, as symptoms and exacerbation rate data were

not collected routinely before 2012. Furthermore, the addition of the symptoms and the number of exacerbations are of unproved value in designing studies on the pathogenesis of COPD.

All patients with COPD were stable, and none of the subjects with COPD were treated with theophylline, antibiotics, antioxidants, mucolytic agents, or glucocorticoids, or any combination thereof, in the month prior to bronchoscopy or lung resection. The study conformed to the Declaration of Helsinki and was approved by the institutional review boards of Istituti Clinici Scientifici Maugeri (protocol p81) and the University Hospital of Ferrara.

#### Other Methods

A detailed description of the lung function and fiberoptic bronchoscopy results; the collection, processing, and immunohistochemical analysis of the bronchial biopsy samples and the peripheral lung (e-Tables 1, 2); and the statistical analysis are provided in e-Appendix 1.

## Results

### Clinical Characteristics of Subjects Providing Bronchial Biopsy and Peripheral Lung Samples

We obtained and studied bronchial biopsy samples from 55 subjects. Thirty-two subjects had mild/moderate or severe stable COPD, 12 were current or

ex-smokers with normal lung function, and 11 were lifelong nonsmokers with normal lung function (Table 1). In addition, we studied peripheral lung tissue from 24 subjects: 12 smokers with mild/moderate stable COPD and 12 smokers with normal lung function (Table 2).

### Measurement of the Inflammatory Cells in the Bronchial Lamina Propria of COPD and Control Subjects

The data obtained from patients with stable COPD by immunohistochemical analysis confirmed previous results<sup>21</sup> showing elevated numbers of CD8<sup>+</sup> T cells, CD68<sup>+</sup> macrophages, and neutrophils in COPD.

### Immunohistochemical Results for TGF- $\beta$ Signaling Pathway Members in the Bronchial Epithelium

No differences in the expression of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ RI, TGF- $\beta$ RII, TGF- $\beta$ RIII, TGF $\beta$ -1/BIGH3, TGIF2, SMAD2, SMAD3, SMAD6, SMAD7, CCN2, LTBP-1, or TRAP-1 were seen in the bronchial epithelia of subjects with COPD compared with nonsmoking control subjects. The number of BAMBI<sup>+</sup> immunostained cells was significantly increased in the bronchial epithelia of subjects with COPD compared with control subjects (Fig 1A-E, Table 3).

### Immunohistochemical Results for TGF- $\beta$ Signaling Pathway Members in the Lamina Propria

No differences in TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ RI, TGF- $\beta$ RII, TGF- $\beta$ RIII, TGFBI/BIGH3, TGIF2, SMAD2, SMAD3, SMAD6, SMAD7, CCN2, or TRAP-1 expression were observed between groups in the lamina propria (Table 3).

The number of TGF- $\beta$ 3<sup>+</sup> immunostained cells was significantly increased in the bronchial lamina propria of patients with mild/moderate COPD and control smokers with normal lung function compared with nonsmoking control subjects (Table 3). However, subjects with severe/very severe COPD had significantly decreased TGF- $\beta$ 3<sup>+</sup> cells compared with control smokers with normal lung function. LTBP-1<sup>+</sup> immunostaining (Table 3) and the number of BAMBI<sup>+</sup> cells were significantly increased in all severities of COPD compared with control subjects (Fig 1A-F, Table 3). No differences in TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ RI, TGF- $\beta$ RII, TGF- $\beta$ RIII, TGFBI/BIGH3, TGIF2, SMAD2, SMAD3, SMAD6, SMAD7, CCN2, or TRAP-1 expression were observed between groups in the lamina propria.

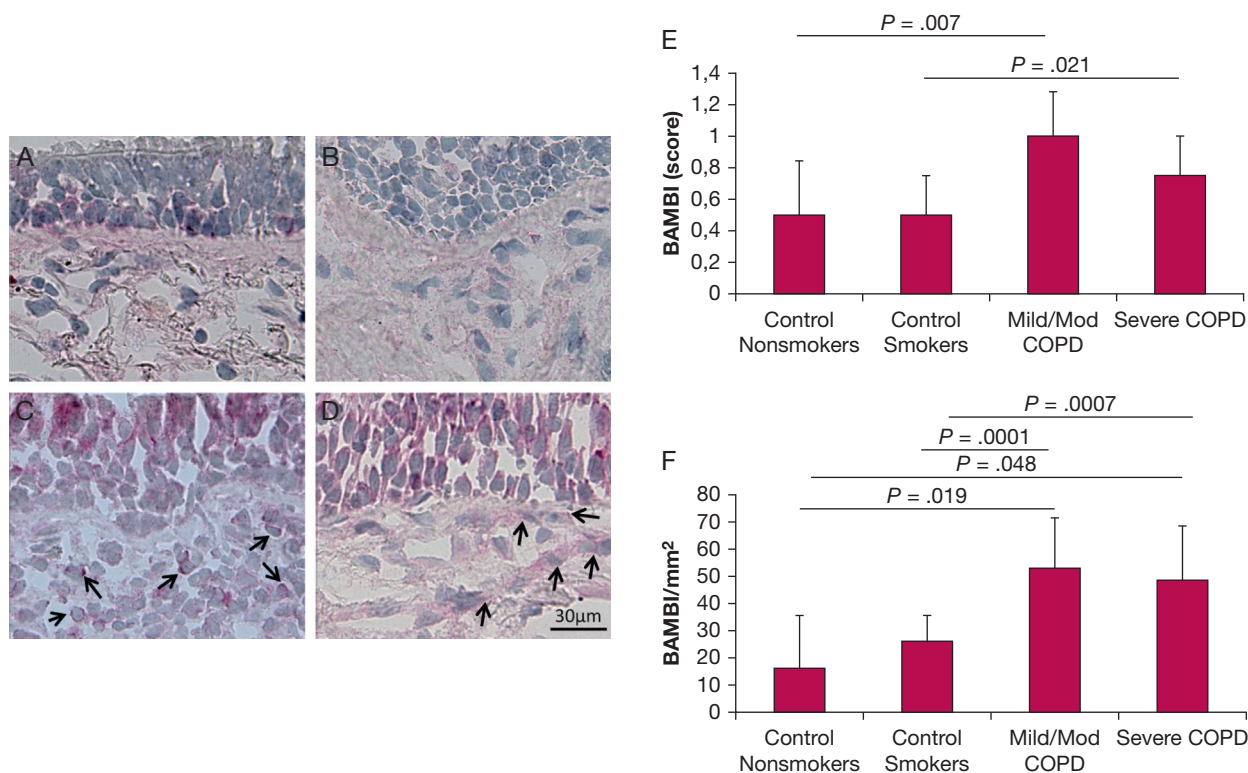


Figure 1 – Photomicrographs showing the bronchial mucosa from (A) control nonsmoker, (B) control healthy smoker with normal lung function, (C) mild/moderate stable COPD, (D) severe/very severe stable COPD immunostained for identification of BAMBI<sup>+</sup> cells (arrows) in the epithelium and bronchial lamina propria. Results are representative of those from 11 nonsmokers, 12 healthy smokers, 14 subjects with mild/moderate COPD, and 18 subjects with severe/very severe COPD. Graphs indicate median (interquartile range) values of (E) BAMBI scored in the epithelium, BAMBI (score), and (F) BAMBI<sup>+</sup> cells quantified in the lamina propria (BAMBI/mm<sup>2</sup>) of the groups of subjects studied. P values were obtained using the Mann-Whitney test for comparison between groups. BAMBI = bone morphogenetic proteins and activin membrane-bound inhibitor.

**TABLE 3 ] Immunohistochemical Quantification of TGF-β Signaling Pathways in Bronchial Biopsy Samples**

| Target   | Nonsmokers With Normal Lung Function | Smokers With Normal Lung Function | Mild/Moderate COPD               | Severe/Very Severe COPD          | Kruskal-Wallis P Value |
|--|--------------------------------------|-----------------------------------|----------------------------------|----------------------------------|------------------------|
| <b>Bronchial epithelium score (0-3)</b>                      |                                      |                                   |                                  |                                  |                        |
| TGF-β1   | 0.25 (0.0-0.75)                      | 0.25 (0.0-0.75)                   | 0.25 (0.0-1.0)                   | 0.37 (0.0-0.75)                  | .945                   |
| TGF-β2   | 0.12 (0.0-1.5)                       | 0.0 (0.0-1.5)                     | 0.0 (0.0-1.5)                    | 0.25 (0.0-2.0)                   | .846                   |
| TGF-β3   | 0.0 (0.0-0.0)                        | 0.0 (0.0-0.0)                     | 0.0 (0.0-0.0)                    | 0.0 (0.0-0.0)                    | n.v.                   |
| TGF-βRI  | 0.0 (0.0-0.25)                       | 0.0 (0.0-0.25)                    | 0.0 (0.0-1.0)                    | 0.0 (0.0-0.25)                   | .661                   |
| TGF-βRII   | 0.5 (0.2-2.0)                        | 0.5 (0.0-3.0)                     | 0.5 (0.0-2.0)                    | 1.0 (0.0-2.0)                    | .405                   |
| TGFβ-RIII  | 0.25 (0.0-0.5)                       | 0.25 (0.0-0.75)                   | 0.25 (0.0-1.0)                   | 0.25 (0.0-0.75)                  | .334                   |
| TGFBI/BIGH3  | 0.0 (0.0-0.0)                        | 0.0 (0.0-0.25)                    | 0.0 (0.0-0.0)                    | 0.0 (0.0-0.25)                   | .971                   |
| TGIF2  | 1.5 (1.25-2.0)                       | 1.5 (0.75-1.75)                   | 1.75 (0.75-2.5)                  | 1.5 (1.0-2.0)                    | .185                   |
| SMAD2  | 1.0 (0.0-2.5)                        | 0.5 (0.0-3.0)                     | 0.0 (0.0-2.0)                    | 1.0 (0.0-2.5)                    | .249                   |
| SMAD3  | 1.0 (0.0-2.0)                        | 0.0 (0.0-3.0)                     | 0.5 (0.0-1.5)                    | 1.0 (0.0-2.5)                    | .468                   |
| SMAD6  | 0.50 (0.25-1.0)                      | 0.50 (0.0-1.5)                    | 0.75 (0.0-1.5)                   | 0.25 (0.25-1.25)                 | .296                   |
| SMAD7  | 0.75 (0.0-2.0)                       | 0.5 (0.0-2.5)                     | 0.35 (0.0-2.5)                   | 0.5 (0.0-2.0)                    | .797                   |
| CCN2   | 1.5 (1.0-1.5)                        | 1.5 (1.0-2.5)                     | 1.75 (1.0-3.0)                   | 1.5 (1.0-2.5)                    | .866                   |
| LTBP-1   | 0.25 (0.0-0.25)                      | 0.0 (0.0-0.25)                    | 0.25 (0.0-0.75)                  | 0.25 (0.0-0.5)                   | .263                   |
| TRAP-1   | 0.0 (0.0-0.0)                        | 0.0 (0.0-0.0)                     | 0.0 (0.0-0.0)                    | 0.0 (0.0-0.0)                    | n.v.                   |
| BAMBI  | 0.5 (0.25-1.25)                      | 0.5 (0.25-0.75)                   | 1.0 (0.25-1.75) <sup>a</sup>     | 0.75 (0.25-1.5) <sup>b</sup>     | .033                   |
| <b>Bronchial lamina propria score (cells/mm<sup>2</sup>)</b> |                                      |                                   |                                  |                                  |                        |
| TGF-β1   | 27.0 (5.0-60.0)                      | 12.5 (0.0-143.0)                  | 16.0 (0.0-64.0)                  | 28.0 (5.0-141.0)                 | .752                   |
| TGF-β2   | 28.0 (14.0-69.0)                     | 13.0 (0.0-37.0)                   | 19.5 (0.0-56.0)                  | 23.0 (0.0-68.0)                  | .211                   |
| TGF-β3   | 0.0 (0.0-13.0)                       | 16.0 (6.0-58.0) <sup>a</sup>      | 10.0 (0.0-39.0) <sup>a</sup>     | 4.5 (0.0-65.0) <sup>b</sup>      | .007                   |
| TGF-βRI  | 5.0 (0.0-52.0)                       | 6.0 (0.0-75.0)                    | 13.0 (0.0-97.0)                  | 5.0 (0.0-73.0)                   | .186                   |
| TGF-βRII   | 74.0 (0.0-225.0)                     | 48.0 (0.0-216.0)                  | 72.5 (8.0-505.0)                 | 45.0 (0.0-376.0)                 | .528                   |
| TGF-βRIII  | 6.0 (4.0-23.0)                       | 6.0 (0.0-32.0)                    | 11.0 (0.0-97.0)                  | 8.0 (5.0-121.0)                  | .612                   |
| TGFBI/BIGH3  | 251.0 (138-484)                      | 304.0 (174-548)                   | 338.0 (218-408)                  | 361.0 (244-468)                  | .142                   |
| TGIF2  | 204 (64-352)                         | 169 (77-277)                      | 177 (77-322)                     | 157 (122-235)                    | .712                   |
| SMAD2  | 181.5 (0.0-750.0)                    | 166.5 (50.0-514.0)                | 51.0 (0.0-960.0)                 | 91.5 (4.0-627.0)                 | .530                   |
| SMAD3  | 77.0 (0.0-690.0)                     | 87.0 (6.0-754.0)                  | 111.0 (0.0-909.0)                | 120.0 (0.0-353.0)                | .822                   |
| SMAD6  | 45.5 (12.0-145.0)                    | 60.5 (12.0-148.0)                 | 83.0 (21.0-134.0)                | 32.0 (9.0-148.0)                 | .143                   |
| SMAD7  | 67.0 (11.0-300.0)                    | 48.5 (0.0-584.0)                  | 96.0 (0.0-520.0)                 | 56.5 (0.0-620.0)                 | .949                   |
| CCN2   | 147.0 (84.0-210.0)                   | 86 (52.0-234.0)                   | 111.0 (55.0-312.0)               | 102.0 (48.0-168.0)               | .768                   |
| LTBP-1   | 3.0 (0.0-13.0)                       | 1.5 (0.0-8.0)                     | 5.0 (0.0-65.0) <sup>b</sup>      | 9.5 (0.0-109.0) <sup>b</sup>     | .017                   |
| TRAP-1   | 0.0 (0.0-6.0)                        | 0.0 (0.0-5.0)                     | 0.0 (0.0-12.0)                   | 0.0 (0.0-5.0)                    | .243                   |
| BAMBI  | 16.0 (7.0-86.0)                      | 26.0 (8.0-35.0)                   | 53.0 (24.0-364.0) <sup>a,b</sup> | 48.5 (16.0-258.0) <sup>a,b</sup> | .0001                  |

Data are expressed as median (range). BAMBI = bone morphogenetic protein and activin membrane-bound inhibitor. CTGF = connective tissue growth factor; LTBP-1 = latent transforming growth factor-β1 binding protein 1; n.v. = no value. SMAD = small mother against decapentaplegic TGFBI = transforming growth factor-β-induced protein; TGIF2 = TGF-β-induced factor 2; TRAP-1 = transforming growth factor-β receptor-associated binding protein. Statistics: The Kruskal-Wallis test was used for multiple comparisons followed by the Mann-Whitney U test for comparison between groups. <sup>a</sup>P < .05, which was significantly different from control nonsmokers. <sup>b</sup>P < .05, which was significantly different from control smokers.

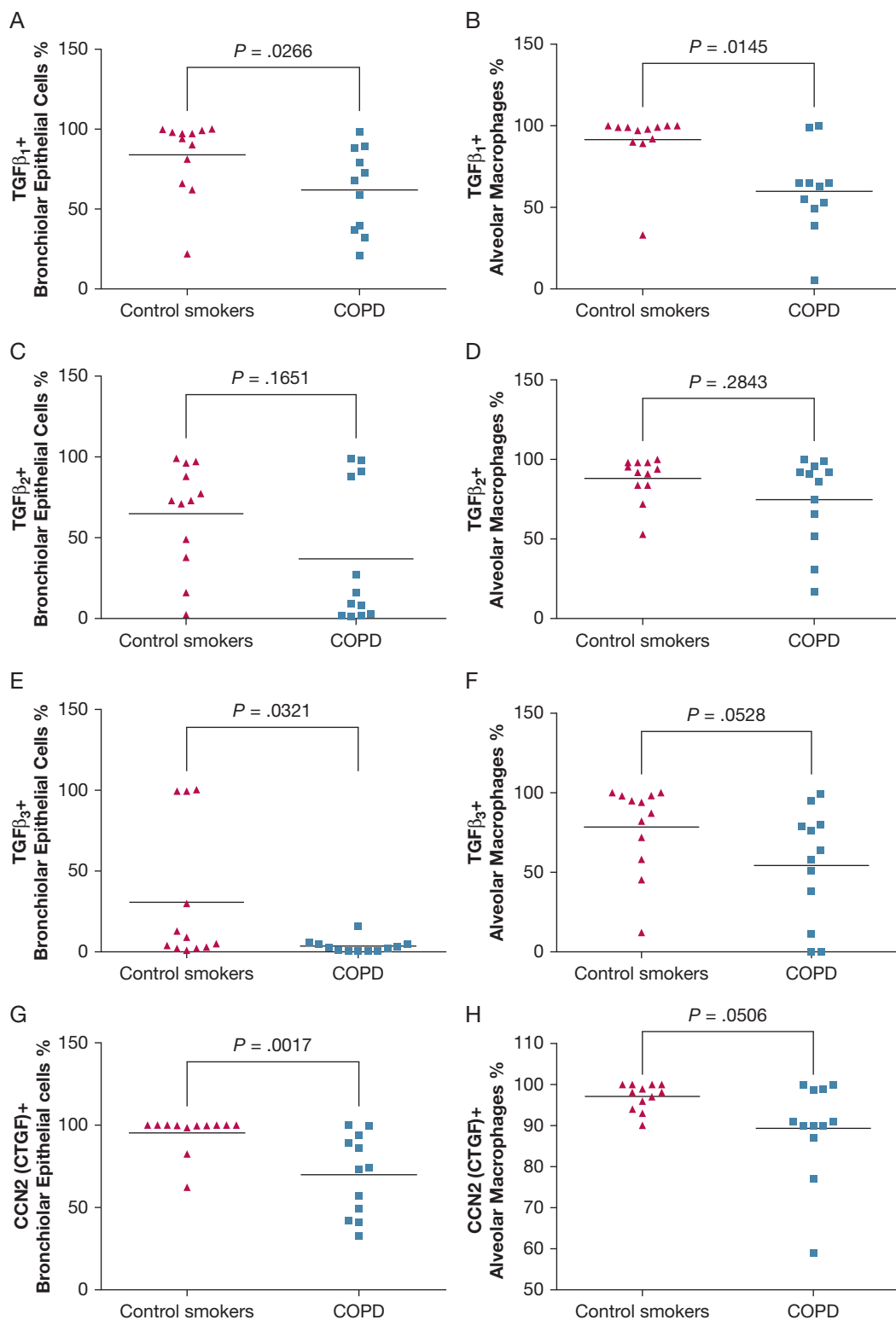


Figure 2 – The percentage of the (A) bronchiolar epithelial and (B) alveolar macrophage cells immunostained for TGF- $\beta_1$  and (G and H) CCN2 (CTGF). Values for (C and D) TGF- $\beta_2$  and (E and F) TGF- $\beta_3$  are shown. Results are representative of those from 12 subjects with stable COPD and 12 control smokers with normal lung function. The Mann-Whitney U test was used for statistical analysis. Exact P values are shown above each graph.

**TABLE 4 ] Immunohistochemical Quantification of TGF- $\beta$  Signaling Pathways in the Peripheral Lung**

| Localization and Antigen                          | Control Smokers     | COPD                | Mann-Whitney <i>U</i> Test <i>P</i> Value |
|---|---------------------|---------------------|---|
| <b>Bronchiolar epithelium (cells, percentage)</b> |                     |                     |   |
| TGF- $\beta$ 1                                    | 95.5 (69.8-98.8)    | 68.0 (37.0-88.0)    | .0266                                     |
| TGF- $\beta$ 2                                    | 73.0 (40.8-94.0)    | 12.5 (2.3-90.3)     | .1651                                     |
| TGF- $\beta$ 3                                    | 7.0 (2.3-81.8)      | 2.5 (0.4-5.0)       | .0321                                     |
| TGF- $\beta$ -RI                                  | 85.0 (80.0-92.3)    | 91.5 (84.0-96.5)    | .1081                                     |
| TGF- $\beta$ -RII (bronchiolar smooth muscle)     | 15.5 (8.3-23.5)     | 11.0 (0.5-20.3)     | .4003                                     |
| TGF- $\beta$ -RIII                                | 0.0 (0.0-0.0)       | 0.0 (0.0-0.0)       | NA  |
| TGIF2 (nuclear)                                   | 3.0 (0.0-21.0)      | 12.5 (7.5-19.8)     | .1623                                     |
| TGIF2 (apical)                                    | 60.0 (34.5-85.3)    | 52.5 (45.5-73.0)    | .9539                                     |
| SMAD2 (nuclear)                                   | 9.0 (4.0-21.5)      | 6.0 (1.3-34.8)      | .7947                                     |
| SMAD2 (cytosolic)                                 | 10.5 (1.5-22.5)     | 1.0 (0.0-7.8)       | .0610                                     |
| SMAD3   | 5.0 (2.3-30.8)      | 15.5 (1.0-45.5)     | .7066                                     |
| SMAD6   | 100.0 (99.8-100.0)  | 100.0 (99.2-100.0)  | .6148                                     |
| SMAD7   | 99.0 (97.3-99.7)    | 99.0 (93.0-99.0)    | .5516                                     |
| TRAP-1 (nuclear)                                  | 5.0 (0.3-12.8)      | 6.0 (0.3-15.5)      | .8612                                     |
| TRAP-1 (cytosolic)                                | 0.0 (0.0-0.0)       | 0.0 (0.0-7.5)       | .4255                                     |
| CCN2  | 100.0 (98.8-100.0)  | 73.5 (43.8-92.8)    | .0017                                     |
| LTBP-1  | 0.0 (0.0-0.0)       | 0.0 (0.0-0.0)       | .8939                                     |
| BAMBI (nuclear)                                   | 55.5 (38.2-75.1)    | 51.3 (40.0-60.3)    | .5833                                     |
| BAMBI (cytosolic)                                 | 4.0 (2.1-6.6)       | 3.5 (1.2-5.8)       | .5031                                     |
| <b>Alveolar macrophages (cells, percentage)</b>   |                     |                     |   |
| TGF- $\beta$ 1                                    | 98.5 (90.5-99.8)    | 63.0 (50.0-66.0)    | .0145                                     |
| TGF- $\beta$ 2                                    | 93.0 (84.0-98.0)    | 88.5 (55.5-95.0)    | .2843                                     |
| TGF- $\beta$ 3                                    | 90.5 (61.5-98.0)    | 61.0 (17.8-79.8)    | .0528                                     |
| TGF- $\beta$ RI                                   | 87.5 (34.0-93.5)    | 85.0 (70.8-97.8)    | .5435                                     |
| TGF- $\beta$ RII                                  | 11.0 (0.0-40.5)     | 29.5 (12.0-47.8)    | .1737                                     |
| TGF- $\beta$ RIII                                 | 18.0 (11.0-63.8)    | 42.5 (20.8-76.0)    | .2850                                     |
| TGIF2 (nuclear)                                   | 7.5 (2.3-23.0)      | 7.5 (1.3-15.8)      | .7501                                     |
| TGIF2 (cytosolic)                                 | 73.5 (45.3-93.3)    | 73.5 (72.3-81.0)    | .9769                                     |
| SMAD2 (nuclear)                                   | 0.0 (0.0-0.0)       | 0.0 (0.0-1.8)       | .3496                                     |
| SMAD2 (cytosolic)                                 | 63.0 (41.0-82.0)    | 60.5 (39.3-85.8)    | .9770                                     |
| SMAD3 (nuclear)                                   | 0.0 (0.0-1.0)       | 0.0 (0.0-1.0)       | .9175                                     |
| SMAD3 (cytosolic)                                 | 39.5 (32.0-68.3)    | 27.0 (10.0-79.8)    | .4024                                     |
| SMAD6   | 100.0 (100.0-100.0) | 100.0 (100.0-100.0) | NA  |
| SMAD7   | 100.0 (100.0-100.0) | 100.0 (100.0-100.0) | .3384                                     |
| TRAP-1 (nuclear)                                  | 14.0 (11.0-19.0)    | 22.5 (1.8-24.0)     | .1558                                     |
| TRAP-1 (cytosolic)                                | 79.0 (65.0-82.8)    | 76.5 (63.0-79.5)    | .3545                                     |
| CCN2  | 98.0 (94.5-100.0)   | 90.5 (87.8-98.9)    | .0506                                     |
| LTBP-1  | 29.5 (15.8-46.5)    | 45.0 (29.5-77.3)    | .1120                                     |
| BAMBI (nuclear)                                   | 53.8 (7.0-66.8)     | 18.5 (10.8-31.1)    | .0832                                     |
| BAMBI (cytosolic)                                 | 32.5 (24.2-40.0)    | 42.5 (26.0-59.5)    | .2038                                     |
| <b>Lung vessels (score)</b>                       |                     |                     |   |
| TGFBI/BIGH3                                       | 1.0 (1.0-1.8)       | 1.0 (1.0-2.0)       | .3132                                     |
| SMAD2   | 1.0 (1.0-2.0)       | 1.5 (1.0-2.8)       | .3440                                     |

Data expressed as median (range). Statistics: The Mann-Whitney *U* test was applied for comparison between groups. Note: when not specified, the positive staining is intended to be nuclear, apart from TGF $\beta$ -RI, in which it is apical in the bronchiolar epithelium and cytosolic in the alveolar macrophages, and TGF- $\beta$ RIII and LTBP-1, for which it is cytosolic. NA = not applicable. See Table 3 legend for expansion of other abbreviations.

### *Immunohistochemical Results of the TGF- $\beta$ Signaling Pathway in the Peripheral Lung*

At variance with the bronchial epithelium, the percentage of TGF- $\beta$ 1<sup>+</sup> (Fig 2A, e-Fig 1), TGF- $\beta$ 3<sup>+</sup> (Fig 2E, e-Fig 2), and CCN2<sup>+</sup> (Fig 2G, e-Fig 3) bronchiolar epithelial cells in the peripheral airways was significantly decreased in patients with COPD compared with control smokers with normal lung function (Table 4). The percentage of TGF- $\beta$ 1<sup>+</sup> alveolar macrophages was decreased in patients with COPD compared with control smokers with normal lung function (Fig 2B, Table 4). There was also a trend for decreased TGF- $\beta$ 3<sup>+</sup> and CCN2<sup>+</sup> immunostained alveolar macrophages in patients with COPD (Figs 2F, 2H, Table 4). No other significant differences were observed between groups for any other molecules studied (Fig 2, Table 4, e-Fig 4).

### *Analysis of Gene Expression Data in Large and Small Airway Epithelial Cells*

We examined the relative expression of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, CCN2, LTBP1, and BAMBI mRNA in epithelial cells from the small (GSE11784) and large airways (GSE37147) of patients with COPD compared with control subjects (e-Table 3) using previously published data sets. These data demonstrated a lack of concordance between protein and mRNA for TGF- $\beta$  pathway members (compare results in e-Table 4 with those in Tables 3 and 4). BAMBI mRNA expression was not different between bronchial epithelial cells from subjects with COPD (n = 30) and healthy smokers (n = 69), although active smoking had a small significant (adjusted  $P = .03$ ) 1.14-fold increase in BAMBI mRNA (e-Table 3). In contrast, LTBP-1 mRNA expression in subjects with COPD was significantly less than that in nonsmokers (adjusted  $P = 8.74 \times 10^{-14}$ ).

In contrast to the reduction in TGF- $\beta$ 1, TGF- $\beta$ 3, and CCN2 protein expression in COPD compared with cells from healthy smokers, there was no difference in TGF- $\beta$ 3 mRNA in small airway epithelial cells, but there was an increase in TGF- $\beta$ 1 and CCN2 mRNA expression (e-Table 3). TGF- $\beta$ 1 mRNA expression was significantly increased at 1.22-fold (adjusted  $P = .00194$ ) and CCN2 was increased by 1.3-fold (adjusted  $P = .0024$ ) in cells from subjects with COPD (n = 36) vs those from healthy nonsmokers (n = 69).

### *Correlations Between Clinical Parameters, Inflammatory Cell Counts, and TGF- $\beta$ Signaling Pathway in Bronchial Biopsy Samples*

There was a significant correlation between the number of cigarettes smoked (pack-years) and the number of

TGF- $\beta$ 3<sup>+</sup> immunostained cells/mm<sup>2</sup> in the bronchial lamina propria when subjects with or without COPD were grouped together (Fig 3A). This correlation was maintained within the COPD group alone (Fig 3B). The numbers of BAMBI<sup>+</sup> immunostained cells/mm<sup>2</sup> in the bronchial lamina propria were significantly correlated with numbers of CD8<sup>+</sup> cells/mm<sup>2</sup> (Fig 3C) and CD68<sup>+</sup> cells/mm<sup>2</sup> (Figure 3D). No other significant correlations were observed between groups for all the other molecules studied.

## **Discussion**

We report here for the first time, to our knowledge, the comprehensive expression and localization of TGF- $\beta$  regulatory proteins in the lower airways of patients with stable COPD compared with control subjects. We observed decreased expression of TGF- $\beta$ 1 and TGF- $\beta$ 3 in the bronchiolar but not bronchial epithelium and of TGF- $\beta$ 1 in alveolar macrophages of patients with stable COPD compared with control smokers with normal lung function. Furthermore, TGF- $\beta$ 3 expression was increased in the bronchial lamina propria of control smokers with normal lung function and mild/moderate stable COPD compared with control nonsmokers and correlated significantly with pack-years. These data suggest that the TGF- $\beta$  signaling is selectively impaired in the small airway epithelia of patients with stable COPD. The expression of the TGF- $\beta$  pseudoreceptor BAMBI was also elevated in the bronchial mucosa of patients with COPD.

Conflicting results have previously been reported regarding the expression of the TGF- $\beta$  signaling pathway in patients with stable COPD in bronchial biopsy samples and peripheral lung (e-Table 4). Our data for TGF- $\beta$ 1 agree with some previous studies<sup>22,23</sup> but not others.<sup>24,25</sup> The latter studies found increased TGF- $\beta$ 1 expression in the small airway and alveolar epithelial cells, but when the control subjects used for the study are classified according to current GOLD criteria using FEV<sub>1</sub>/FVC ratios, many would be reclassified as having COPD.<sup>24</sup> Another study reported increased TGF- $\beta$ 1 protein for both COPD and control smokers compared with nonsmoking subjects.<sup>26</sup> A more recent study found increased release of total TGF- $\beta$ 1 from bronchial epithelium in vitro obtained from patients with COPD compared with control subjects without any significant differences in active TGF- $\beta$ 1 release.<sup>27</sup> Finally, another study found no significant differences in BAL TGF- $\beta$ 1 levels between patients with COPD and control subjects.<sup>28</sup> These apparent discrepancies may be explained by incomplete clinical



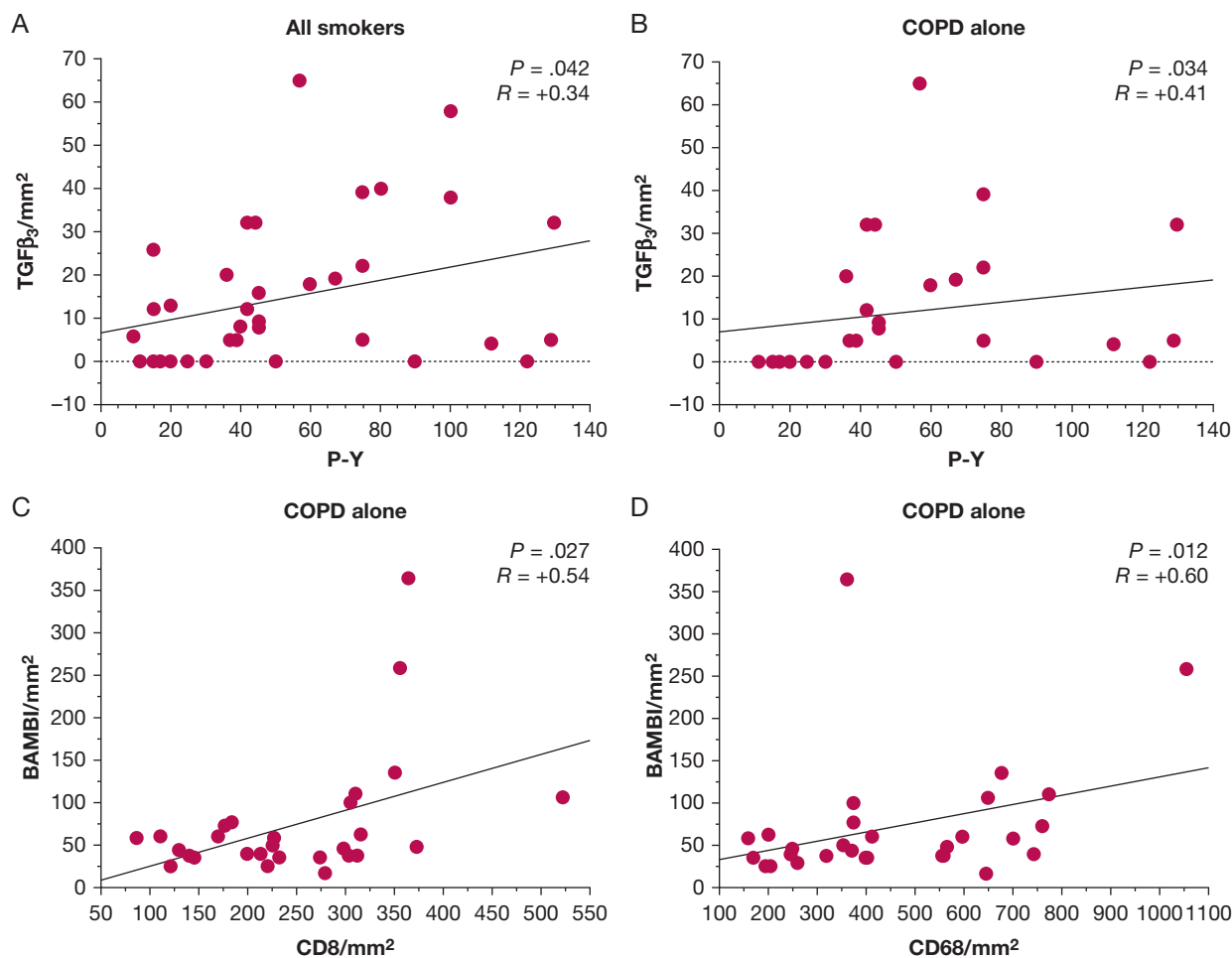


Figure 3 – Regression analysis between pack-years and number of TGF-β3<sup>+</sup> cells infiltrating the bronchial lamina propria in (A) all smokers (with and without COPD) and (B) patients with COPD alone. In the latter patients, there is a significant positive correlation between the number of BAMBI<sup>+</sup> cells in the bronchial lamina propria and those of (C) CD8<sup>+</sup> and (D) CD68<sup>+</sup> cells. Correlation coefficients were calculated by using the Spearman rank method. See Figure 1 legend for expansion of abbreviations.

features or the selectivity of the primary anti-TGF-β1 antibodies used, or both.<sup>29,30</sup>

Llinàs et al<sup>31</sup> observed decreased TGF-β1 mRNA expression in the peripheral lungs of patients with severe stable COPD compared with control nonsmoking subjects, and in agreement with our present data, Kokturk et al<sup>32</sup> found no difference in TGF-β1 immunohistochemical expression in the bronchial biopsy samples from patients with stable COPD and control nonsmoking subjects. Vignola et al<sup>33</sup> demonstrated increased TGF-β1 immunostaining in the bronchial biopsy samples of patients with chronic bronchitis compared with control young nonsmoking subjects (mean age, 46 years). However, these patients had a mean FEV<sub>1</sub> of 56% to 90% predicted, but only nine subjects were considered to have COPD using unspecified criteria.<sup>33</sup> Hence, these differences render the comparison of our results with the previous studies difficult.<sup>33,34</sup> Decreased

expression of TGF-β1 in the small airways in COPD shown here and its critical role in regulating self-tolerance<sup>5</sup> may explain the autoimmunity seen in some patients with COPD.<sup>6,7</sup> The complex signaling control points within the TGF-β activation pathway may enable targeted treatment of this complication.

TGF-β3 expression was increased in the bronchial lamina propria of patients with COPD and control smokers compared with control nonsmoking subjects, although TGF-β3<sup>+</sup> cells were decreased in patients with severe/very severe COPD compared with control smokers. There was a significant correlation between pack-years and the number of TGF-β3<sup>+</sup> immunostained cells/mm<sup>2</sup> in the bronchial lamina propria. To our knowledge, this is the first report of TGF-β3 protein expression and localization in the lower airways of patients with stable COPD and is in keeping with the results of decreased TGF-β3 mRNA expression in the peripheral lung of patients with more

severe COPD<sup>31,35</sup> and of increased TGF- $\beta$ 3 mRNA expression in the peripheral lung of smokers compared with nonsmokers.<sup>31</sup> This concordance was not observed with our present mRNA results.

TGF- $\beta$ 3 protein release from bronchial epithelium *in vitro* and BAL TGF- $\beta$ 3 levels were similar between patients with COPD and control subjects.<sup>27,28</sup> We were unable to find any significant differences between patients with stable COPD and control subjects in TGF- $\beta$ 2 and TGF- $\beta$ R expression and localization in the lower airways. In addition, we could not confirm the decreased TGF- $\beta$ RI protein expression observed in the peripheral lungs of patients with moderate stable COPD compared with control subjects in previous studies.<sup>23,31</sup> This discrepancy may be explained by differences in anti-TGF- $\beta$ RI antibody used. In contrast, our results on TGF- $\beta$ RII protein expression are in agreement with the data from the same study<sup>23</sup> and with the mRNA data from Llinàs et al.<sup>31</sup>

To our knowledge, we have provided the first data on TGF- $\beta$ RIII protein expression, showing its low immune expression in the lower airways of patients with stable COPD and control subjects. Single nucleotide polymorphisms are likely to be associated with decreased TGF- $\beta$ RIII function and are linked to an increased risk of pulmonary emphysema developing.<sup>36</sup> This area requires further research.

We found no significant differences between patients with stable mild/moderate COPD and control subjects (including smokers with normal lung function) in SMAD2, SMAD3, SMAD6, and SMAD7 expression and localization in the lower airways, confirming previous studies.<sup>23,37,38</sup> In contrast, reduced SMAD6 and SMAD7 mRNA expression was reported in bronchial biopsy samples from patients with stable COPD,<sup>39</sup> as well as decreased SMAD7 mRNA in the bronchial epithelium<sup>40</sup> and decreased SMAD mRNA expression<sup>41</sup> and SMAD protein expression in the peripheral lung.<sup>42</sup> These discrepancies may be explained by the lack of concordance between mRNA and protein, as we described in our study comparing gene expression data, or by the potential different pathogenic pathways behind the onset of COPD vs pulmonary emphysema.<sup>43-45</sup> Overall, these data suggest limited involvement of SMAD signaling in the pathogenesis of lower airway inflammation and damage in patients with stable mild/moderate COPD.

We also demonstrated decreased CCN2 (CTGF) expression in the bronchiolar epithelium but not in the

bronchial mucosa of patients with stable COPD compared with control smokers with normal lung function. Conflicting results have previously been reported for CCN2 mRNA expression in patients with COPD.<sup>31,46</sup> TGF- $\beta$ 1 induces CCN2 expression in fibroblasts *in vitro*,<sup>47</sup> suggesting that the decreased expression of both TGF- $\beta$ 1 and CCN2 observed in the bronchiolar epithelium of the patients in our study with stable COPD may be related. Lung specimens from subjects with a solitary peripheral neoplasm were used in the present study, raising the question as to whether lung tumors may influence the results. The presence of similar pathologic conditions in smokers and patients with COPD and the large number of studies already published examining inflammatory markers and cytokine pathways in similar sets of subjects support the use of these groups of patients for peripheral lung studies.

LTBP-1 is the only LTBP that both interacts with latent TGF- $\beta$ s<sup>48</sup> and is predominantly expressed in the lungs.<sup>49-53</sup> LTBP-1 immunostaining was increased in the bronchial lamina propria of patients with COPD compared with control smokers with normal lung function. LTBP-1 may activate TGF- $\beta$ 3, but the concomitant upregulation of TGF- $\beta$ 3 observed in the bronchial lamina propria in our study is smoking related and not disease related, suggesting the presence of alternative mechanisms regulating the expression of these two molecules. In fact, a substantial amount of LTBP-1 can be secreted by cells without being bound to latent TGF- $\beta$ s, and TGF- $\beta$ -independent functions for LTBP-1 need to be determined.<sup>54</sup>

We observed a marked increase in the expression of the TGF- $\beta$  pseudoreceptor BAMBI in the bronchial mucosa but not in the peripheral airways of patients with stable COPD compared with control subjects in keeping with previous studies in COPD peripheral lung.<sup>55</sup> There was a positive correlation between the number of BAMBI<sup>+</sup> and CD8<sup>+</sup> and CD68<sup>+</sup> cells. These data are in keeping with the enhanced plasma BAMBI levels recently described in stable COPD that positively correlated with the blood Th17/regulatory T cells (Treg) ratio.<sup>56</sup> In a mouse model of autoimmune arthritis, BAMBI deficiency protected mice against the development of disease by modulating Th17/Treg differentiation.<sup>57</sup> This may account for the autoimmune and the Th17/Treg imbalance that we and others have previously described in the bronchial mucosa of patients with stable COPD.<sup>6,7,58</sup>

The mechanisms regulating BAMBI expression are poorly understood. In vitro, BAMBI expression can be upregulated by TGF- $\beta$ 1.<sup>19</sup> However, the discrepancy observed between TGF- $\beta$ 1 and BAMBI expression in the different compartments of the lower airways suggest that non-TGF- $\beta$ 1-dependent pathways could be involved in BAMBI upregulation in stable COPD. As a potential limitation of this study, we did not apply multiple corrections in the statistical analysis of differences between groups for our “ex vivo” data of the lower airways. In fact, applying multiple corrections can lead to false-negative findings, but by

not applying them, findings might be false positive. We were confident that applying a multiple test (analysis of variance or Kruskal-Wallis) followed by a “restricted” test analyzing differences between groups could be sufficiently stringent for identification of true differences.

In conclusion, the reported differences in TGF- $\beta$ 1 and BAMBI expression may contribute to the pathogenesis of stable COPD creating a microenvironment facilitating local autoimmune responses associated with COPD.

## Acknowledgments

**Author contributions:** A. D. S., G. C., I. M.A., F. L. M. R., and F. C., contributed to study design, interpretation of data, and writing the manuscript. P. J. B., K. F. C., P. B., P. M., P. R., S. P., Y. G., G. G., and B. B. contributed to critical revision of the manuscript. C. S., I. G., P. C., P. B., M. C., A. D. S., G. C., and A. P. contributed to the production of the data and accuracy of the data analysis.

**Financial/nonfinancial disclosures:** The authors have reported to *CHEST* the following: F. L. M. R. has served on the advisory boards of AstraZeneca, Novartis, and Mundipharma. He has served as a consultant for AstraZeneca, Boehringer Ingelheim, Chiesi, Mundipharma, Malesci-Guidotti, Novartis, and Teva and has received grants from AstraZeneca, Boehringer Ingelheim, Chiesi, and GlaxoSmithKline. A. P. reports grants, personal fees, or reimbursement for travel expenses from AstraZeneca, Chiesi, Boehringer Ingelheim, GlaxoSmithKline, Menarini, Merck Sharp & Dohme, Mundipharma, Novartis, Teva, Sanofi, and Zambon. None declared (A. D. S., C. S., I. G., P. C., P. B., M. C., P. M., P. R., G. G., F. C., S. P., Y. G., K. F. C., B. J. B., I. M. A., B. B., G. C.).

**Role of sponsors:** The sponsor had no role in the design of the study, the collection and analysis of the data, or the preparation of the manuscript.

**Additional information:** The e-Appendix, e-Figures, and e-Tables can be found in the Supplemental Materials section of the online article.

## References

- Wang X, Inoue S, Gu J, et al. Dysregulation of TGF- $\beta$ 1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. *Proc Natl Acad Sci U S A*. 2005;102(44):15791-15796.
- Gao C, Maeno T, Ota F, et al. Sensitivity of heterozygous  $\alpha$ 1,6-fucosyltransferase knock-out mice to cigarette smoke-induced emphysema: implication of aberrant transforming growth factor- $\beta$  signaling and matrix metalloproteinase gene expression. *J Biol Chem*. 2012;287(20):16699-16708.
- Willems-Widyastuti A, Alagappan VK, Arulmani U, et al. Transforming growth factor- $\beta$ 1 induces angiogenesis in vitro via vascular endothelial growth factor production in human airway smooth muscle cells. *Indian J Biochem Biophys*. 2011;48(4):262-269.
- Kasahara Y, Tudor RM, Cool CD, Lynch DA, Flores SC, Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Respir Crit Care Med*. 2001;163(3 pt 1):737-744.
- Sanjabi S, Oh SA, Li MO. Regulation of the immune response by tgf- $\beta$ : from conception to autoimmunity and infection. *Cold Spring Harb Perspect Biol*. 2017;9(6).
- Kirkham PA, Caramori G, Casolari P, et al. Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2011;184(7):796-802.
- Caramori G, Casolari P, Barczyk A, Durham AL, Di Stefano A, Adcock I. COPD immunopathology. *Semin Immunopathol*. 2016;38(4):497-515.
- Santibañez JF, Quintanilla M, Bernabeu C. TGF- $\beta$ /TGF- $\beta$  receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)*. 2011;121(6):233-251.
- Zhang YE. Non-Smad Signaling pathways of the TGF- $\beta$  family. *Cold Spring Harb Perspect Biol*. 2017;9(2).
- Groneberg DA, Witt H, Adcock IM, Hansen G, Springer J. Smads as intracellular mediators of airway inflammation. *Exp Lung Res*. 2004;30(3):223-250.
- Gatza CE, Oh SY, Blobel GC. Roles for the type III TGF- $\beta$  receptor in human cancer. *Cell Signal*. 2010;22(8):1163-1174.
- Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signaling by BMP and TGF- $\beta$ . *Nat Cell Biol*. 2002;4(8):599-604.
- Robertson IB, Horiguchi M, Zilberberg L, Dabovic B, Hadjiolova K, Rifkin DB. Latent TGF- $\beta$ -binding proteins. *Matrix Biol*. 2015;47:44-53.
- Todorovic V, Rifkin DB. LTBP3, more than just an escort service. *J Cell Biochem*. 2012;113(2):410-418.
- Hinz B. The extracellular matrix and transforming growth factor- $\beta$ 1: tale of a strained relationship. *Matrix Biol*. 2015;47:54-65.
- Roberts AB, Russo A, Felici A, Flanders KC. Smad3: a key player in pathogenetic mechanisms dependent on TGF- $\beta$ . *Ann N Y Acad Sci*. 2003;995:1-10.
- Wotton D, Lo RS, Swaby LA, Massagué J. Multiple modes of repression by the Smad transcriptional corepressor TGIF. *J Biol Chem*. 1999;274(52):37105-37110.
- Shangguan L, Ti X, Krause U, et al. Inhibition of TGF- $\beta$ /Smad signaling by BAMBI blocks differentiation of human mesenchymal stem cells to carcinoma-associated fibroblasts and abolishes their protumor effects. *Stem Cells*. 2012;30(12):2810-2819.
- Sekiya T, Oda T, Matsuura K, Akiyama T. Transcriptional regulation of the TGF- $\beta$  pseudoreceptor BAMBI by TGF- $\beta$  signaling. *Biochem Biophys Res Commun*. 2004;320(3):680-684.
- Thapa N, Lee BH, Kim IS. TGF $\beta$ 1/pbetaig-h3 protein: a versatile matrix molecule induced by TGF- $\beta$ . *Int J Biochem Cell Biol*. 2007;39(12):2183-2194.
- Di Stefano A, Caramori G, Barczyk A, et al. Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD. *Thorax*. 2014;69(6):516-524.
- Pons AR, Sauleda J, Noguera A, et al. Decreased macrophage release of TGF- $\beta$  and TIMP-1 in chronic obstructive pulmonary disease. *Eur Respir J*. 2005;26(1):60-66.

23. Zandvoort A, Postma DS, Jonker MR, et al. Altered expression of the Smad signaling pathway: implications for COPD pathogenesis. *Eur Respir J*. 2006;28(3):533-541.
24. de Boer WI, van Schadewijk A, Sont JK, et al. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 1998;158(6):1951-1957.
25. Zanini A, Chetta A, Saetta M, et al. Bronchial vascular remodelling in patients with COPD and its relationship with inhaled steroid treatment. *Thorax*. 2009;64(12):1019-1024.
26. Takizawa H, Tanaka M, Takami K, et al. Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med*. 2001;163(6):1476-1483.
27. Gohy ST, Detry BR, Lecocq M, et al. Polymeric immunoglobulin receptor down-regulation in chronic obstructive pulmonary disease. Persistence in the cultured epithelium and role of transforming growth factor- $\beta$ . *Am J Respir Crit Care Med*. 2014;190(5):509-521.
28. Forslund H, Yang M, Mikko M, et al. Gender differences in the T-cell profiles of the airways in patients with COPD associated with clinical phenotypes. *Int J Chron Obstruct Pulmon Dis*. 2016;12:35-48.
29. Aubert JD, Dalal BI, Bai TR, Roberts CR, Hayashi S, Hogg JC. Transforming growth factor beta 1 gene expression in human airways. *Thorax*. 1994;49(3):225-232.
30. Jurukovski V, Dabovic B, Todorovic V, Chen Y, Rifkin DB. Methods for measuring TGF- $\beta$  using antibodies, cells, and mice. *Methods Mol Med*. 2005;117:161-175.
31. Llinàs L, Peinado VI, Ramon Goñi J, et al. Similar gene expression profiles in smokers and patients with moderate COPD. *Pulm Pharmacol Ther*. 2011;24(1):32-41.
32. Kokturk N, Tatlicioglu T, Memis L, Akyurek N, Akyol G. Expression of transforming growth factor beta1 in bronchial biopsies in asthma and COPD. *J Asthma*. 2003;40(8):887-893.
33. Vignola AM, Chanez P, Chiappara G, et al. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med*. 1997;156(2 pt 1):591-599.
34. Matěj R, Vašáková M, Kukal J, Sterclová M, Olejár T. Higher TGF- $\beta$  with lower CD124 and TSLP, but no difference in PAR-2 expression in bronchial biopsy of bronchial asthma patients in comparison with patients with COPD. *Appl Immunohistochem Mol Morphol*. 2014;22(7):543-549.
35. Gosselink JV, Hayashi S, Elliott WM, et al. Differential expression of tissue repair genes in the pathogenesis of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2010;181(12):1329-1335.
36. Hersh CP, Hansel NN, Barnes KC, et al. Transforming growth factor-beta receptor-3 is associated with pulmonary emphysema. *Am J Respir Cell Mol Biol*. 2009;41(3):324-331.
37. Eurlings IM, Dentener MA, Cleutjens JP, et al. Similar matrix alterations in alveolar and small airway walls of patients with COPD. *BMC Pulm Med*. 2014;14:90.
38. Zandvoort A, Postma DS, Jonker MR, Noordhoek JA, Vos JT, Timens W. Smad gene expression in pulmonary fibroblasts: indications for defective ECM repair in COPD. *Respir Res*. 2008;9:83.
39. Springer J, Scholz FR, Peiser C, Gronenberg DA, Fischer A. SMAD-signaling in chronic obstructive pulmonary disease: transcriptional down-regulation of inhibitory SMAD6 and 7 by cigarette smoke. *Biol Chem*. 2004;385(7):649-653.
40. Ezzie ME, Crawford M, Cho JH, et al. Gene expression networks in COPD: microRNA and mRNA regulation. *Thorax*. 2012;67(2):122-131.
41. Campbell JD, McDonough JE, Zeskind JE, et al. A gene expression signature of emphysema-related lung destruction and its reversal by the tripeptide GHK. *Genome Med*. 2012;4(8):67.
42. Leppäranta O, Myllärniemi M, Salmenkivi K, Kinnula VL, Keski-Oja J, Koli K. Reduced phosphorylation of the TGF-Beta signal transducer Smad2 in emphysematous human lung. *COPD*. 2009;6(4):234-241.
43. D'Anna SE, Asnaghi R, Caramori G, et al. High-resolution computed tomography quantitation of emphysema is correlated with selected lung function values in stable COPD. *Respiration*. 2012;83(5):383-390.
44. Galbán CJ, Han MK, Boes JL, et al. Computed tomography-based biomarker provides unique signature for diagnosis of COPD phenotypes and disease progression. *Nat Med*. 2012;18(11):1711-1715.
45. McDonough JE, Yuan R, Suzuki M, et al. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *N Engl J Med*. 2011;365(17):1567-1575.
46. Ning W, Li CJ, Kaminski N, et al. Comprehensive gene expression profiles reveal pathways related to the pathogenesis of chronic obstructive pulmonary disease. *Proc Natl Acad Sci U S A*. 2004;101(41):14895-14900.
47. Pohlert D, Brenmoehl J, Löffler I, et al. TGF-beta and fibrosis in different organs—molecular pathway imprints. *Biochim Biophys Acta*. 2009;1792(8):746-756.
48. Saharinen J, Keski-Oja J. Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-beta. *Mol Biol Cell*. 2000;11(8):2691-2704.
49. Davis MR, Andersson R, Severin J, et al. Transcriptional profiling of the human fibrillin/LTBP gene family, key regulators of mesenchymal cell functions. *Mol Genet Metab*. 2014;112(1):73-83.
50. Giltay R, Kostka G, Timpl R. Sequence and expression of a novel member (LTBP-4) of the family of latent transforming growth factor-beta binding proteins. *FEBS Lett*. 1997;411(2-3):164-168.
51. Morén A, Olofsson A, Stenman G, et al. Identification and characterization of LTBP-2, a novel latent transforming growth factor-beta-binding protein. *J Biol Chem*. 1994;269(51):32469-32478.
52. Saharinen J, Taipale J, Monni O, Keski-Oja J. Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4. *J Biol Chem*. 1998;273(29):18459-18469.
53. Penttinen C, Saharinen J, Weikkolainen K, Hyytiäinen M, Keski-Oja J. Secretion of human latent TGF-beta-binding protein-3 (LTBP-3) is dependent on co-expression of TGF-beta. *J Cell Sci*. 2002;115(pt 17):3457-3468.
54. Rifkin DB. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem*. 2005;280(9):7409-7412.
55. Drömann D, Rupp J, Rohmann K, et al. The TGF-beta-pseudoreceptor BAMB1 is strongly expressed in COPD lungs and regulated by nontypeable Haemophilus influenzae. *Respir Res*. 2010;11:67.
56. Zhang JC, Chen G, Chen L, et al. TGF- $\beta$ /BAMB1 pathway dysfunction contributes to peripheral Th17/Treg imbalance in chronic obstructive pulmonary disease. *Sci Rep*. 2016;6:31911-31921.
57. Postigo J, Iglesias M, Álvarez P, et al. Bone Morphogenetic protein and activin membrane-bound inhibitor, a transforming growth factor  $\beta$  rheostat that controls murine Treg Cell/Th17 Cell differentiation and the development of autoimmune arthritis by reducing interleukin-2 signaling. *Arthritis Rheum*. 2016;68(6):1551-1562.
58. Di Stefano A, Caramori G, Gnemmi I, et al. T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol*. 2009;157(2):316-324.