



Clinical Trial Paper

Effect of bronchial thermoplasty on structural changes and inflammatory mediators in the airways of subjects with severe asthma

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ABSTRACT

Background: Bronchial thermoplasty (BT) is a novel technique used in the treatment of subjects with severe refractory asthma. Radiofrequency is provided to airway walls during bronchoscopy in order to reduce airway remodeling. Several clinical studies have reported an improvement in subjects' symptoms following BT. However, how BT affects the airway architectures and inflammatory mediators in the airways has not been yet fully elucidated.

Methods: Fourteen subjects with severe asthma were recruited in this study according to the criteria of ATS severe asthma definition. The study subjects undertook bronchial biopsy during the bronchoscopy procedure at baseline and 6 weeks after the initial BT treatment. The obtained samples were stained with antibodies for α -smooth muscle actin (α -SMA); protein gene product (PGP) 9.5, a specific nerve marker; von Willebrand factor (vWF), a marker for blood vessels; interleukin-17A (IL-17A) and transforming growth factor- β 1 (TGF- β 1).

Results: The expression of α -SMA and PGP9.5 were significantly reduced post-BT. There was no significant difference in the number of blood vessels between baseline and post-BT. In addition, BT did not affect the production of IL-17A and TGF- β 1 in the airways. The changes in the expression of α -SMA and PGP9.5 had no significant correlation with the improvement of pulmonary function.

Conclusion: and Clinical Relevance: This study suggests that BT reduces airway smooth muscle mass and the airway innervation without affecting vasculature and the production of inflammatory mediators in the airways of subjects with severe asthma.

1. Introduction

Airway irreversible structural changes in asthma, termed airway remodeling, are key pathological features of severe asthma [1]. Remodeling in asthma is comprised of subepithelial fibrosis, goblet cell hyperplasia, angiogenesis, epithelial alterations and increased mass of airway smooth muscle [2,3]. Subjects with severe asthma have poor control of asthma despite high doses of inhaled corticosteroids and other medications [4]. Bronchial thermoplasty (BT) is an emerging technique as an alternative catheter-based treatment for severe asthma that delivers thermal energy to the airway wall to ablate airway smooth

muscle (ASM) mass [5,6]. BT has been used clinically to improve asthma-related quality of life and reduce asthma exacerbations in clinical trials [7–9]. Several studies of BT have demonstrated a significant reduction in ASM area in the airways of subjects with severe asthma [10–12], thus potentially explaining in part the mechanisms contributing to effectiveness of BT.

Since temperature-controlled radiofrequency waves can potentially alter airway structures other than ASM, additional mechanisms might contribute to the observed beneficial effects of BT. Few studies have investigated the effects of BT on architectures other than ASM in the airways of subjects with severe asthma [13–15]. Of the alternative

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mechanisms contributing to effectiveness of BT the number of blood vessels and lymphatic vessels warrants consideration as well since increased blood vessel number and vascular area are observed and changes of vasculature can contribute to airway wall engorgement and reduced airway caliber in the airways of asthmatic subjects [16,17]. Furthermore, Denner et al. addressed the effects of BT on the production of asthma-related mediators demonstrating reduction of transforming growth factor-beta (TGF- β)1 and TNF-related apoptosis-inducing ligand (TRAIL) in bronchoalveolar lavage fluids (BALF) of subjects post-BT [11]. IL-17, which also plays a pivotal role in neutrophilic airway inflammation in severe asthma, was undetected in BAL. In this respect, to date, the effect of BT on the cells producing these asthma-related mediators in the airways has not been investigated.

The current study aimed to investigate how BT affects other airway architectures such as nerves and blood vessels, and the expression of inflammatory mediators such as TGF- β 1 and IL-17A in the airways of subjects with severe persistent asthma. To address this, we performed a detailed immunohistochemistry analysis to compare the expression of these architectures and cytokines in the samples at baseline with those obtained at 6 weeks post-BT and correlated them with clinical data of subjects.

2. Methods

2.1. Study subjects

Fourteen adult subjects aged 38–68 with severe persistent asthma, taking STEP 4 or STEP 5 treatment based on the Global Initiative for Asthma (GINA) [18], were recruited to the present study between July 1st, 2013 and Jun 30th, 2014 (Table 1). Four subjects were from Montreal Chest Institute of McGill University Health Center (Group 1) and 10 subjects were from Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval (Group 2). Some of study subjects in group 2 had been included in a previously published study [15]. There was no significant difference of baseline characteristics between both groups except the numbers of subjects with seasonal allergy and the use of omalizumab (Table 1). Control status of asthma at baseline and post-BT was evaluated by Asthma Control Questionnaire (ACQ) and Asthma Quality Life of Questionnaire (AQLQ) in group 1 and by the Asthma Control Scoring System (ACSS) [19] in group 2. This study was approved by the Institutional Review Board of the McGill University Health Center and Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval.

2.2. BT procedure

BT was performed with the Alair System (Boston Scientific, Natick, MA) under moderate sedation, as previously described [15]. On the morning of the procedure, subjects were assessed for stability of their illness and absence of symptoms of respiratory tract infection. The procedures were postponed if the post-bronchodilator FEV₁ was less than 80% of the subject's prior best. The subjects took prednisone 50 mg daily 4 days before the procedure. Methylprednisolone 40 mg was given intravenously before the procedure, and the subjects continued taking prednisone 50 mg daily for the following 3 days. All subjects underwent 3 sessions of BT to treat all accessible airways, except the right middle lobe at intervals of at least 3 weeks.

2.3. Bronchial biopsies

Biopsy specimens were obtained at the first and second divisions of lobar bronchi. At the first bronchoscopy, 4 biopsies were obtained from the lateral segment of the left lower lobe (untreated lobe, baseline). At the last bronchoscopy, 4 more biopsies were taken from the posterior segment of the previously treated right lobe.

Biopsies were immediately placed into 4% freshly thawed

Table 1

Baseline characteristics of the subjects.

	Group 1 ^a (N = 4)	Group 2 ^b (N = 10)	Group 1 + 2 (N = 14)
Age (years)	48.3 $\pm$ 9.5	50.9 $\pm$ 13.4	50.1 $\pm$ 12.1
Sex (Male/Female) (n)	3/1	6/4	9/5
Smoking status (current/ex/non) (n)	0/0/4	1/5/4	1/5/8
seasonal allergies, n (%)	1 (25)	7 (70)	8 (57.1)
Height (cm)	165.5 $\pm$ 3.7	167.3 $\pm$ 11.3	166.8 $\pm$ 9.6
Weight (kg)	81.0 $\pm$ 11.7	80.5 $\pm$ 14.8	80.6 $\pm$ 13.6
Body Mass Index (kg/ m ²)	29.7 $\pm$ 5.5	28.8 $\pm$ 4.8	29.1 $\pm$ 4.8
Duration of asthma symptoms			
< 5 years, n (%)	0 (0)	2 (20)	2 (14.3)
5–10 years, n (%)	0 (0)	2 (20)	2 (14.3)
11–20 years, n (%)	0 (0)	1 (10)	1 (7.1)
> 20 years, n (%)	4 (100)	5 (50)	9 (64.3)
Medications			
ICS/LABA, n (%)	4 (100)	10 (100)	14 (100)
Leukotriene antagonists, n (%)	1 (25)	5 (50)	6 (42.9)
Oral corticosteroids, n (%)	1 (25)	3 (30)	4 (28.6)
LAMA	1 (25)	6 (30)	7 (50)
Omalizumab	0 (0)	3 (30)	3 (21.4)
Daily dose of ICS (FP equivalent)			
< 500 μ g, n (%)	1 (25)	0 (0)	1 (7.1)
501–1000 μ g, n (%)	3 (75)	2 (20)	5 (35.7)
1001–1500 μ g, n (%)	0 (0)	6 (60)	6 (42.9)
> 1500 μ g n (%)	0 (0)	2 (20)	2 (14.3)
Asthma control status			
ACQ score	2.25 $\pm$ 0.99	N/A	N/A
AQLQ score	4.93 $\pm$ 1.77	N/A	N/A
ACSS ^c	N/A	65.6 $\pm$ 24.4	N/A
Pulmonary function test			
FEV ₁ (L)	1.84 $\pm$ 0.63	2.19 $\pm$ 0.59	2.09 $\pm$ 0.60
%FEV ₁ predicted, (%)	61 $\pm$ 25.9	67 $\pm$ 12.3	65.3 $\pm$ 16.3

Abbreviations: ICS, inhaled corticosteroids; LABA, long-acting β 2-agonist; LAMA, long-acting muscarinic antagonist; FP, fluticasone propionate; ACQ, Asthma Control Questionnaire; AQLQ, Asthma Quality Life of Questionnaire; ACSS, Asthma Control Scoring System; FEV₁, forced expiratory volume in 1 s; N/A, not applicable.

^a Study subjects in group 1 from the Montreal Chest Institute of McGill University Health Center.

^b Study subjects in group 2 from Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval.

^c The number of study subjects in group 2 with available ACSS (n = 9).

paraformaldehyde, kept at 4 °C for a maximum of 24 h, and embedded in paraffin blocks. The changes at 6 weeks post-BT were examined by immunostaining.

2.4. Immunohistochemistry

Tissue sections were deparaffinized in xylene and dehydrated in ethanol series. Slides were washed in PBS for 5 min and processed for antigen retrieval using citrate buffer. The sections were permeabilized with Triton-X 100 for 15 min. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS for 30 min. Sections were then washed in PBS, blocked using DAKO universal blocking reagent for 60 min at room temperature. Sections were incubated overnight at 4 °C with primary goat anti-IL-17A antibody (1:50 dilution; R&D systems, Minneapolis, MN, USA). The primary antibody was replaced by non-immune serum for negative controls. After incubation with biotinylated secondary antibodies, sections were washed 3 times with PBS, incubated with streptavidin-horse radish peroxidase complex, and then developed using diaminobenzidine substrate (DAB) according to the

manufacturer's instructions. Slides were stained with mouse anti-TGF- β 1 (1:100 dilution; R&D systems, Minneapolis, MN, USA) using a BenchMark automated immunostainer (Ventana, Tuscon, AZ, USA). Nucleus was stained blue with Gill II hematoxylin.

2.5. Immunofluorescence

After the process of deparaffinization and antigen retrieval as described above, tissue sections were permeabilized with 0.1% Tween 20 in PBS. Tissue sections were blocked with blocking reagent and incubated overnight at 4 °C with rabbit anti- α -smooth muscle actin (1:500; Abcam, Cambridge, MA), mouse anti-PGP 9.5 (1:100; Abcam), rabbit anti-von Willebrand factor (1:200; Dako) and mouse anti-D2-40 (1:200; Dako) followed by donkey anti-rabbit Alexa fluor 488 antibody or goat anti-mouse Alexafluor 555 antibody (Invitrogen, Burlington, ONT) for 1 h. Diluent without primary antibodies was used as control. After washing with PBS, cell nucleus was stained with Hoechst 33342 (AnaSpec, Fremont, CA) for 10 min and mounted with PermaFluor (Thermo scientific, Fremont, CA). Images were captured with a confocal microscope (LSM780; Carl Zeiss Microscopy, Jena, Germany) and analyzed using ImageJ (National Institutes of Health, Bethesda, MD) software. ASM and nerve area were expressed as percentage of α -ASM- or PGP 9.5-positive area over whole airway area in the section, calculated using ImageJ software.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5 for windows. Data are expressed as median (interquartile range) except those obtained with purified alveolar macrophages which represent means + 1 SEM. Multiple means were compared using the Kruskal-Wallis test and post hoc pairwise comparisons were performed using the Mann-Whitney *U* test. Correlation between changes of clinical parameters, including FEV₁, ACQ, AQLQ and ACSS, changes of ASM area, PGP9.5 expression, and numbers of blood, lymphatic vessels, TGF- β 1- and IL-17A-positive cells were analyzed with Spearman's correlation coefficient.

3. Results

3.1. Changes of airway smooth muscle area and PGP9.5 expression in the airways after bronchial thermoplasty

The ASM area labeled by α -SMA was 7.39% (5.28–8.49%) at baseline. At 6 weeks post-BT, the amount of ASM was significantly reduced to 2.41% (1.97–2.96%; $p = 0.0002$). The level of PGP 9.5 expressions in the airways was 0.60% (0.39–1.23%) at baseline, and significantly reduced to 0.32% (0.17–0.51%) at 6 weeks post-BT ($p = 0.0009$; Fig. 1).

3.2. Changes of numbers of blood vessels and lymphatic vessels in the airways after bronchial thermoplasty

Next, we double-immunostained the sections with the antibodies for vWF and D2-40 to confirm that vWF was selectively expressed on blood vessels, not lymphatic vessels. There was no co-localization of vWF with D2-40 in the airways. At baseline, the numbers of blood vessels labeled by vWF and lymphatic vessels labeled by D2-40 were 120.43 per mm² (91.44–142.78 per mm²) and 8.86 per mm² (5.06–16.06 per mm²), respectively. BT had no significant effects on the numbers of blood vessels: 86.40 per mm² (79.97–125.10 per mm²; $p = 0.0676$) and lymphatic vessels 16.78 per mm² (9.23–29.63 per mm²; $p = 0.1272$) at 6 weeks post-BT (Fig. 2).

3.3. Changes of production of TGF- β 1 and IL-17A in the airways after bronchial thermoplasty

We were able to analyze TGF- β 1 expression on tissue sections from 10 subjects. The total number of TGF- β 1-positive cells was 1709.74 per mm² (1332.30–1989.57 per mm²) at baseline. No significant change in immunoreactive cells was observed at 6 weeks post-BT: 1903.54 per mm² (1794.86–2058.73 per mm²; $p = 0.2754$). The numbers of immunoreactive cells in the epithelial and subepithelial areas were 3442.35 per mm² (3245.00–4550.75 per mm²) and 1440.81 per mm² (1026.28–1729.62 per mm²), respectively, at baseline. The number of immunoreactive cells was not significantly altered by BT at 6 weeks: 3365 per mm² (2547.48–4128.33 per mm²; $p = 0.6250$) and 1495 per mm² (1211.33–1603.38 per mm²; $p = 0.1934$), respectively (Fig. 3A and C). The IL-17A-positive cells in total, epithelial and subepithelial areas were 16.06 per mm² (3.63–23.46 per mm²), 0 per mm² (0–24.51 per mm²) and 9.60 per mm² (2.99–23.29 per mm²), respectively. BT did not affect the numbers of IL-17A expressing cells at 6 weeks after treatment: 20.05 per mm² (0–36.68 per mm²), 0 per mm² (0–0 per mm²) and 19.30 per mm² (0–33.72 per mm²), respectively (Fig. 3B and C).

3.4. Clinical effect on pulmonary function, medication and quality of life

No significant improvement of FEV₁ and FEV₁% predicted was observed at 6 weeks post-BT. We investigated the correlation between the changes of FEV₁ and those of pathological markers, and there was no significant correlation between the change of FEV₁ and the reduction in ASM area and PGP9.5 (Fig. 4A and B). The change in numbers of blood and lymphatic vessels did not correlate with the change in pulmonary function neither (data not shown).

All subjects in group 1 and 9 subjects in group 2 were followed for one year post-BT. One subject in group 2 died during follow-up. Four subjects in the current study required regular oral corticosteroids (OCS) at baseline whereas one subject in group 2 discontinued OCS at one year post-BT. Daily dose of OCS with one subject in group 2 was reduced from 10 mg to 7.5 mg. Daily doses of inhaled corticosteroids (ICS) with four subjects in group 2 were reduced during follow-up. One subject in group 1 discontinued use of long-acting muscarinic antagonists post-BT. Changes of medication were not significantly related with the degree of BT-induced changes of airway structural markers and those of inflammatory mediators.

Annual exacerbation frequencies in groups 2 were significantly reduced in 7 subjects post-BT ($p = 0.0469$). In group 1, we assessed ACQ and AQLQ score in three of the four subjects for one year post-BT. Only one subject met clinically significant improvement of ACQ (decrease greater than 0.5 point) and AQLQ score was improved (increase greater than 0.5 point) in another subject. In group 2, ACSS scores were increased in 6 subjects, reduced in 1 subject and not changed in 1 subject, at one year post-BT. Two subjects could not be followed-up (one subject died and ACSS was not available for one subject at baseline). In group 2, there was a significant correlation between reduction of ASM area and change of ACSS score ($r = -0.727$, $p = 0.0412$, Fig. 4C). In both groups, other clinical parameters including ACQ and AQLQ were not correlated with the changes of structural marker expression and changes of mediator productions.

4. Discussion

In this study, we investigated the short term effects of BT on pathological changes of airways from severe asthmatic subjects who received prednisolone, showing that BT significantly reduced the expression of α -smooth muscle actin and PGP9.5-positive cells, confined to neural and neuroendocrine cells. BT had no significant effect on the number of blood vessels and lymphatic vessels in the airways. BT did not affect either the expression of IL-17A and TGF- β 1 in the airways or

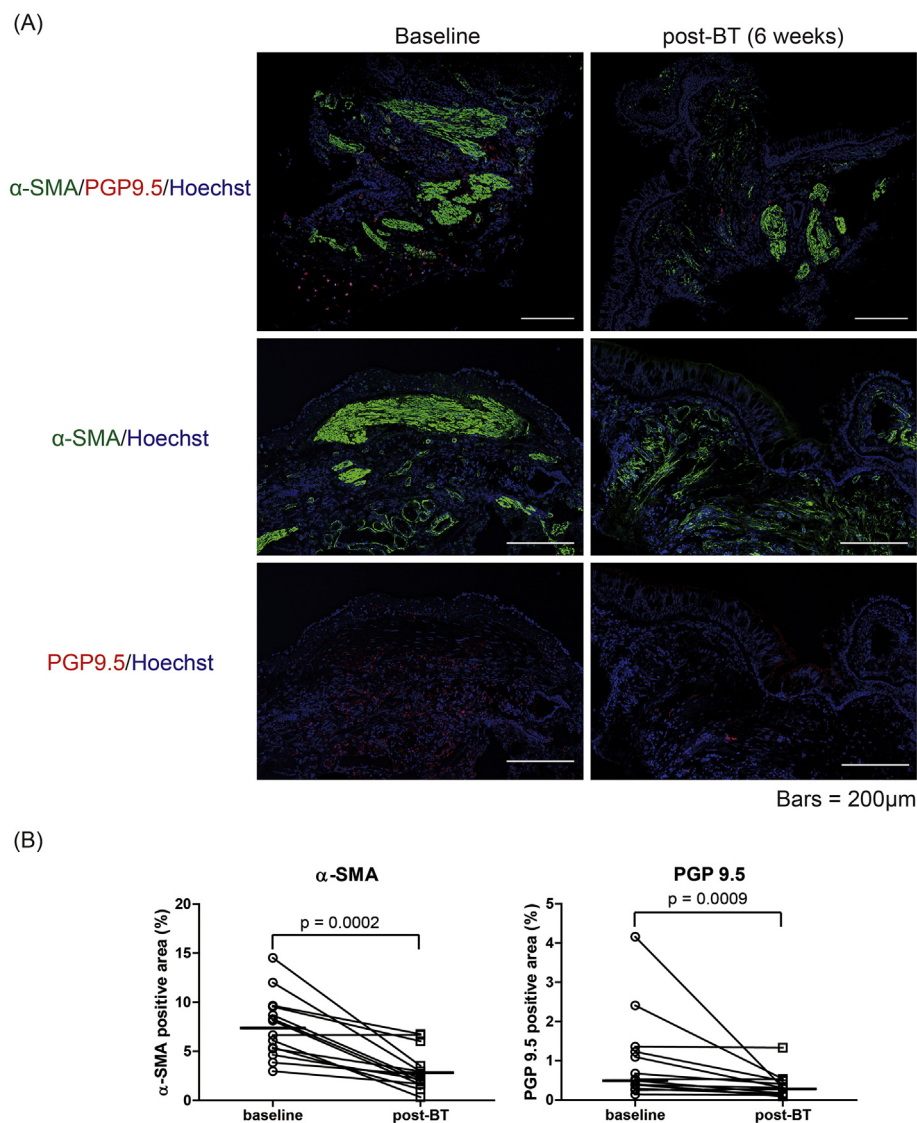


Fig. 1. Effect of bronchial thermoplasty (BT) on expressions of α -smooth muscle actin (SMA) and PGP9.5 in subjects with severe asthma. **(A)** Representative confocal microscopic images from bronchial sections stained with α -SMA (green) and PGP9.5 (red), and nuclear counterstain Hoechst33342 (blue) at baseline (left panels) and 6 weeks post-BT (right panels). Images of whole biopsy area (top panels) and magnified (bottom panels). **(B)** Comparisons of α -SMA (left panel) and PGP9.5 (right panel)-positive area at baseline and post-BT. The areas are expressed as percentage of α -SMA or PGP9.5 positive area over the total biopsy area. Scale bars, 200 μ m.

pulmonary function of the subjects. These data further extend our previous results [15] and suggest that BT-induced reduction of airway smooth muscle mass and nerves in the airways could account for the beneficial effects of BT.

Although a significant reduction in ASM mass after BT has been demonstrated in the airways of subjects with severe asthma in several studies [10,12,20], only a few of them have investigated the effects of BT on architectures other than ASM [10,11,13–15]. Chakir et al. demonstrated that BT decreased type I collagen deposition in the airways [15]. Recent studies have reported also a reduction in basement membrane thickening and the nerve fibers in airways post-BT [13,14]. Our results, showing a significant reduction in the number of epithelial and subepithelial PGP9.5-positive cells in the airways of asthmatic subjects post-BT, expand upon these data, suggesting that BT affects the amount of nerves in the airways of subjects with asthma as early as at 6 weeks. Thus, the nerve reduction could be an important additional mechanism to mediate the beneficial effects of BT in severe asthma.

The autonomic nervous system plays a central role in regulating smooth muscle tone in airways and dysregulation may contribute to the pathogenesis of asthma [21]. Increase in airway smooth muscle tone

may augment the responses to other bronchoconstrictive mediators [22]. Chronic cough, a major symptom of asthmatic subjects, is suggested to arise from hypersensitivity of airway nerves [23,24]. Nerves also modulate the tone of airway smooth muscle, inducing bronchospasm in subjects with asthma [25]. Because neuropeptides secreted by neurons, such as substance P and calcitonin gene-related peptide, reportedly cause neurogenic inflammation and modulate immune function [26], reduction of nerves may improve neuropeptide-associated airway inflammation in asthma. However, further study is needed to clarify the effect of BT on the production of neuropeptides by neurons and other types of cells.

A significant increase in the number of vessels and/or percent of vascular area in the airways of subject with asthma has been observed in comparison to healthy controls [27]. Furthermore, increased vascularization has been shown to correlate with airflow limitation and bronchial hyperresponsiveness [28]. These observations suggest that the reduction in vascularization in the airway may have a beneficial effect on the clinical course of asthma. In contrast to blood vessels, lymphangiogenesis is impaired in the asthmatic airways [29] and the distribution of lymphatic vessels is decreased in the airways of fatal

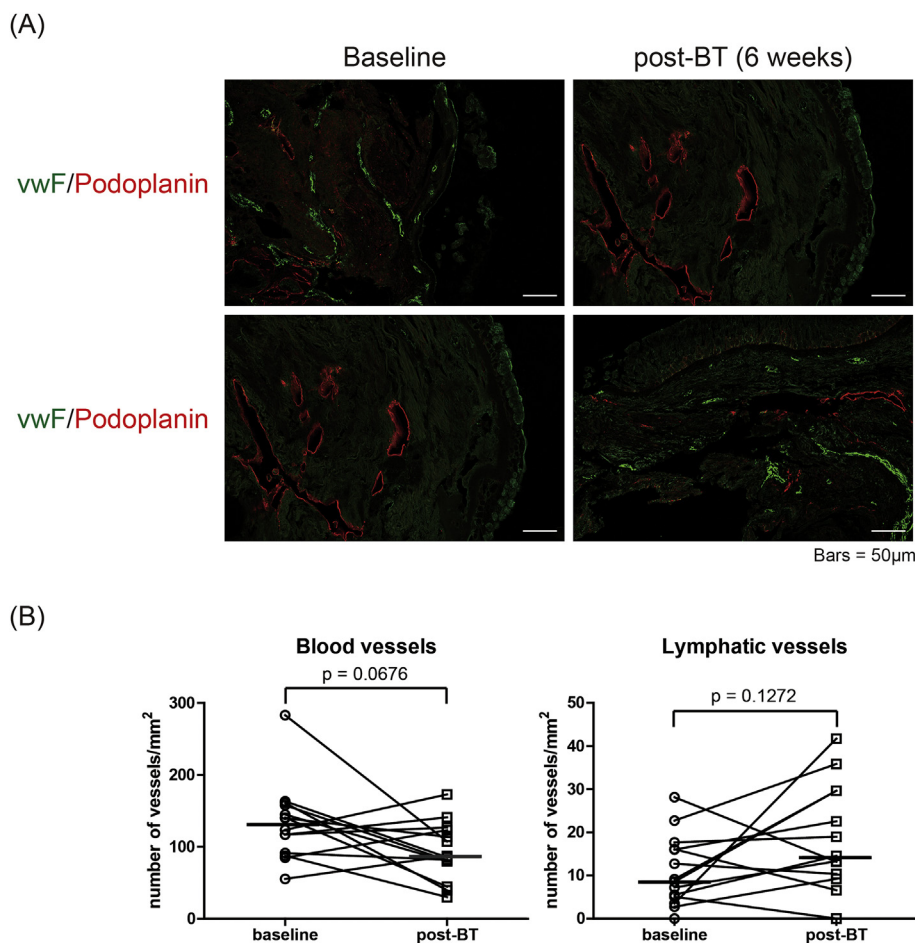


Fig. 2. Effect of BT on blood vessels and lymphatic vessels in airways of subjects with severe asthma. (A) Representative confocal microscopic images from bronchial sections stained with von Willebrand factor (vwF) (green) for blood vessels and podoplanin (red) for lymphatic vessels, and nuclear counterstain Hoechst33342 (blue) at baseline (left panels) and 6 weeks post-BT (right panels). (B) Comparisons of numbers of blood vessels labeled with vwF (left panel) and those of lymphatic vessels labeled with podoplanin (right panel) between baseline and post-BT. Scale bars, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

asthmatics [30]. However, the current study demonstrated that the number of blood and lymphatic vessels in the airways was not changed post-BT, confirming the previous results by Pretolani et al., also showing that BT had no significant effect on the density of blood and lymphatic vessels [13].

BT is expected to be an alternative therapeutic strategy of severe asthma for systemic corticosteroids. At the inflammatory mediators levels related to airway inflammation and fibrotic response, a recent study has reported a decrease in the bronchoalveolar lavage fluid of TGF-β and CCL5 levels 3 and 6 weeks post-BT, whereas IL-4, IL-5, and IL-17 levels were unaffected [11]. Although type I collagen deposition and basement membrane thickening in the asthmatic airways were reportedly decreased with BT [13,15], the present study demonstrated no significant effect of BT on the production of IL-17A and TGF-β1 in the airways of subjects with severe asthma. These data suggest that BT may have the potential to directly reduce the structural component of airway remodeling without any effect on the upstream responses or mechanisms.

In this study, there was a negative correlation between the improvement of ACSS and reduction in ASM area (Fig. 4C). ASM area was expressed as percentage of α-ASM-positive area to avoid variability and inter-observer variations. Contrary to the expectation that the subjects whose ASM mass was largely reduced by BT would show the biggest improvement in their asthma control, the current data indicated that the subjects whose reduction of ASM area was relatively small showed greater improvement of ACSS. As baseline ASM area of those subjects was smaller (data not shown), even a small reduction of ASM might contribute to the improvement of asthma control status. An alternative possibility is that the mechanism of improvement is not strongly dependent on ASM mass. Severe asthmatic subjects may benefit from BT

as early as possible before substantial airway remodeling has been established. Changes of the other structural markers, including PGP 9.5, had no significant effect on improvement of asthma control status.

The limitation of this study is the short interval (6 weeks) of bronchial biopsy between baseline and post-BT. In usual clinical settings, it is difficult to perform bronchoscopy to obtain bronchial biopsy samples early after the last procedure of BT treatment. A short interval might explain why we did not find correlations between BT-induced structural changes and clinical improvement. BT might exert its effect on airway architecture prior to improvement in clinical features. Although we have confirmed a persistence of ASM ablation at 27 months [20], long-term studies are needed to look at other asthma airway characteristics, including airway vascularization, lymphangiogenesis and inflammatory mediators. Another limitation of this study is its small sample size. Although the current study did not quite reach statistical significance for a significant effect of BT on the bronchial vasculature, there was a strong trend for a reduction ($p = 0.0676$), suggesting that the study may have been under-powered to detect the difference. The sample size in the study by Pretolani et al. was also small (15 subjects) [13] and a larger study is needed to clarify whether BT affects the vasculature.

In conclusion, BT selectively reduces ASM mass and nerves in the airways without any significant alterations of the numbers of blood and lymphatic vessels, and the production of inflammatory and profibrotic mediators. As BT bestows clinical benefits on some subjects with severe asthma, it is important to identify the precise mechanisms by which BT affects subjects with severe asthma so that we can prospectively identify the responders to BT.

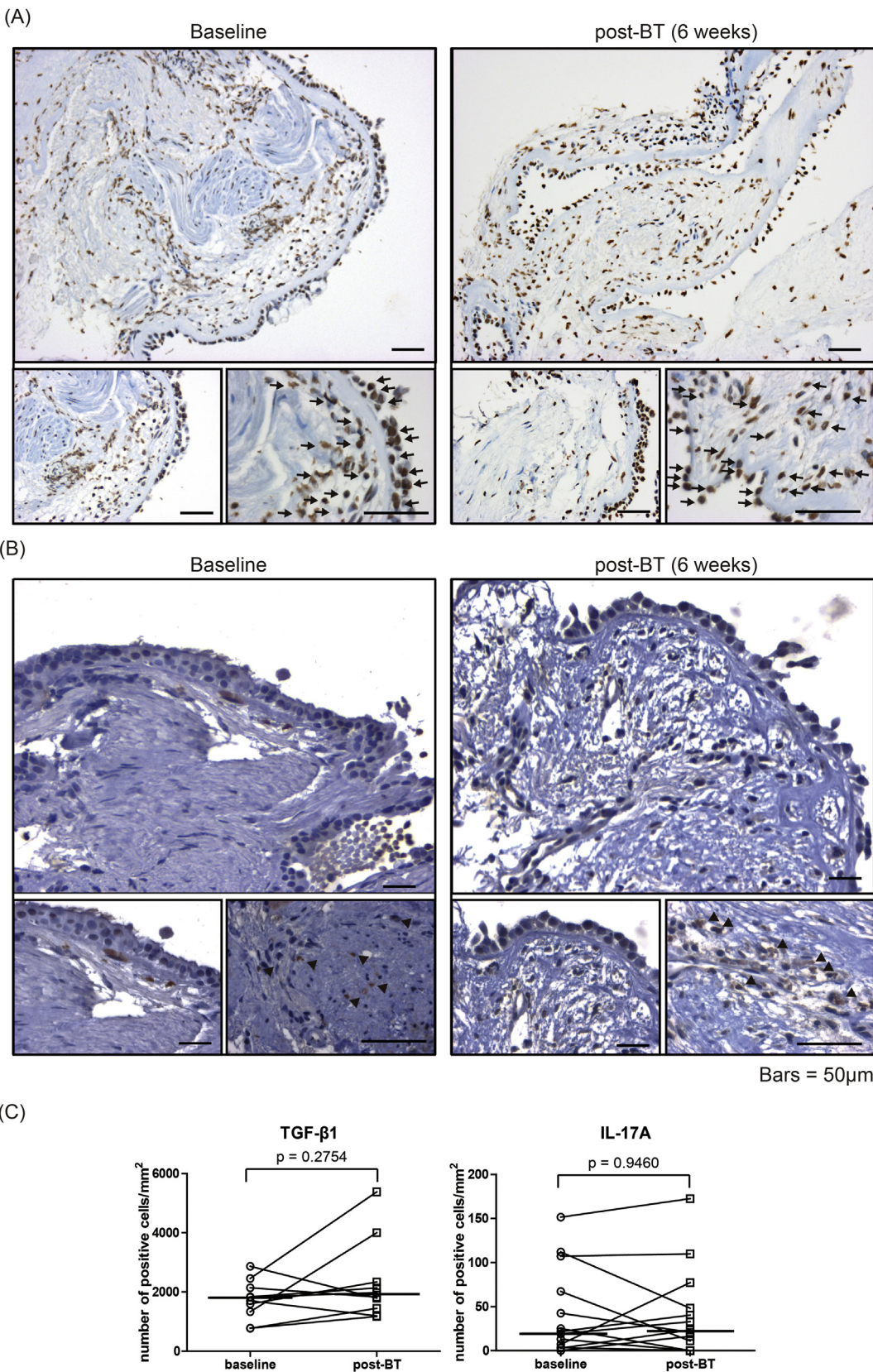


Fig. 3. Effect of BT on TGF-β1 and IL-17A expressions in airways of subjects with severe asthma. Representative microscopic images of TGF-β1 (A) and IL-17A (B) positive cells at baseline (left panels) and 6 weeks post-BT (right panels). Arrow indicates TGF-β1-positive cells and arrowhead indicates IL-17A-positive cells (C) Comparisons of numbers of TGF-β1 (left panel)- and IL-17A (right panel)-positive cells at baseline and post-BT. Scale bars, 50 µm.

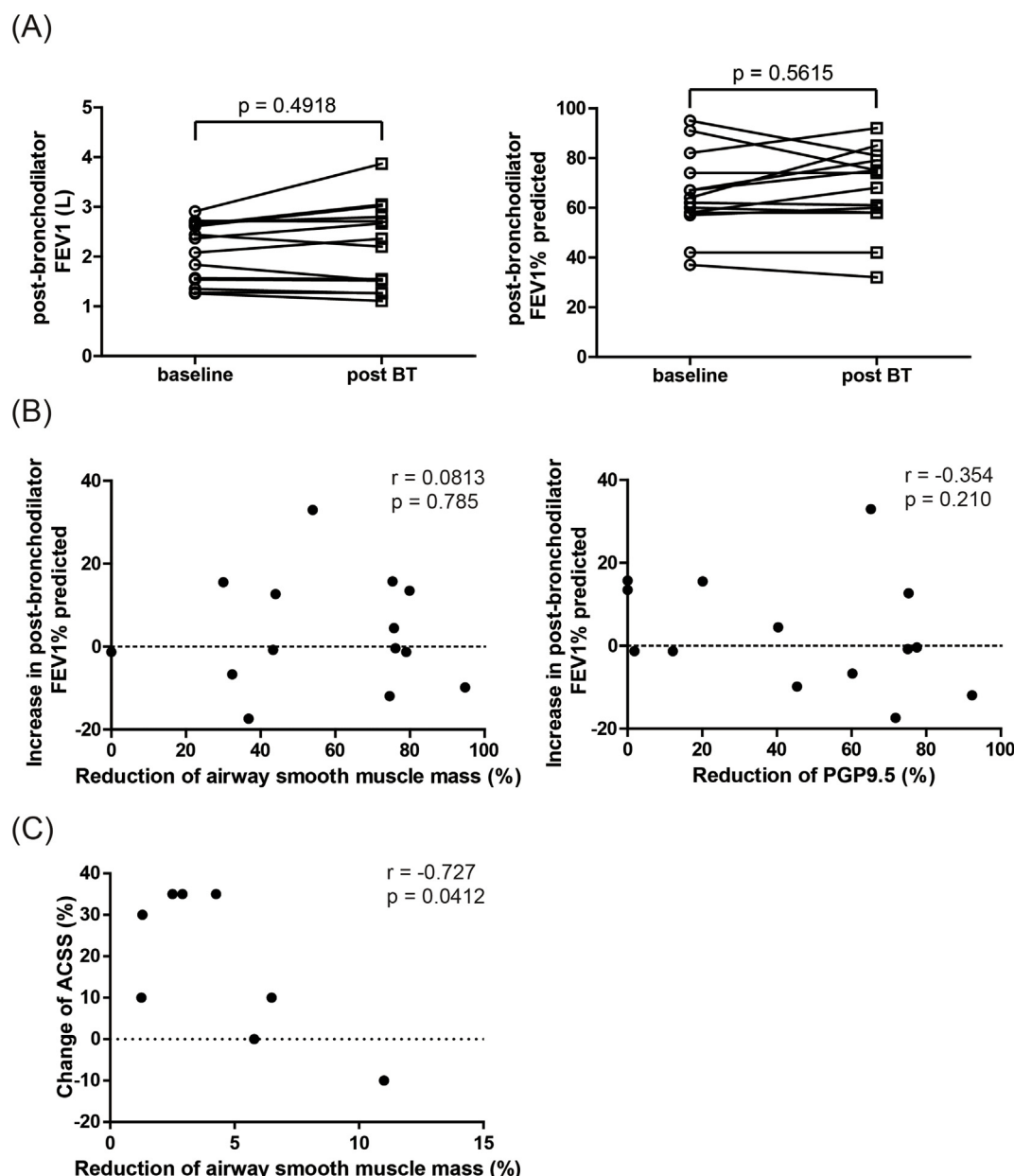


Fig. 4. Effect of BT on pulmonary function. **(A)** Changes of FEV₁ (left panel) and FEV₁% predicted (right panel) at baseline and 6 weeks post-BT. **(B)** Correlations between reduction of smooth muscle mass (left panel) or PGP9.5 expression (right panel) and increase in FEV₁. FEV₁, forced expiratory volume in 1 s. **(C)** Correlation between reduction of smooth muscle mass and changes of ACSS.

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