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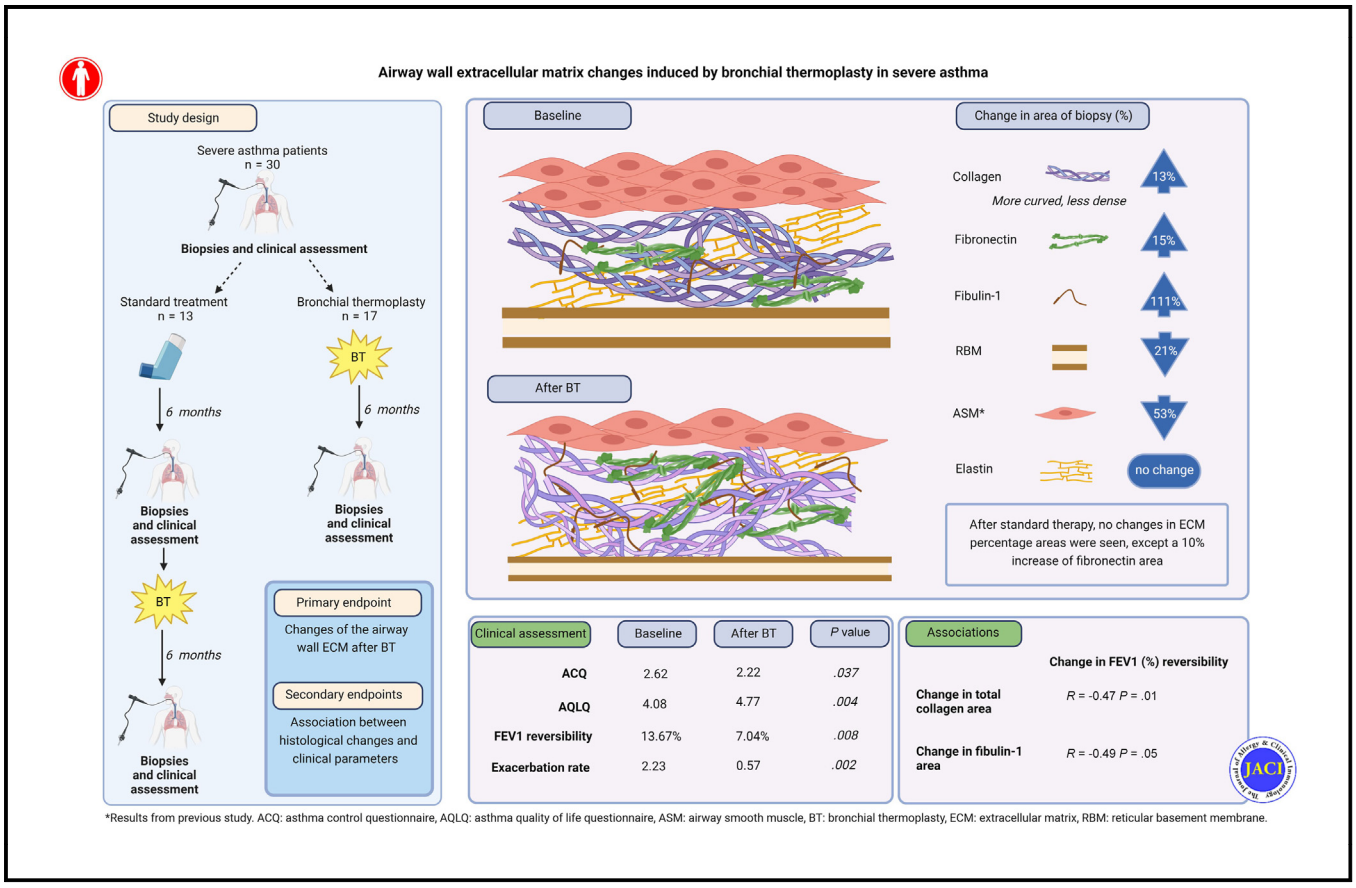
GRAPHICAL ABSTRACT



Capsule summary: This study demonstrates that besides airway smooth muscle mass reduction, bronchial thermoplasty induces extracellular matrix reorganization and stabilization of the airway wall.

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Background: Airway remodeling is a prominent feature of asthma, which involves increased airway smooth muscle mass and altered extracellular matrix composition. Bronchial thermoplasty (BT), a bronchoscopic treatment for severe asthma, targets airway remodeling.

Objective: We sought to investigate the effect of BT on extracellular matrix composition and its association with clinical outcomes.

Methods: This is a substudy of the TASMA trial. Thirty patients with severe asthma were BT-treated, of whom 13 patients were treated for 6 months with standard therapy (control group) before BT. Demographic data, clinical data including pulmonary function, and bronchial biopsies were collected. Biopsies at BT-treated and nontreated locations were analyzed by histological and immunohistochemical staining. Associations between histology and clinical outcomes were explored.

Results: Six months after treatment, it was found that the reticular basement membrane thickness was reduced from 7.28 μm to 5.74 μm (21% relative reduction) and the percentage area of tissue positive for collagen increased from 26.3% to 29.8% (13% relative increase). Collagen structure analysis revealed a reduction in the curvature frequency of fibers. The percentage area positive for fibulin-1 and fibronectin increased by 2.5% and 5.9%, respectively (relative increase of 124% and 15%). No changes were found for elastin. The changes in collagen and fibulin-1 negatively associated with changes in FEV_1 reversibility.

Conclusions: Besides reduction of airway smooth muscle mass, BT has an impact on reticular basement membrane thickness and the extracellular matrix arrangement characterized by an increase in tissue area occupied by collagen with a less dense fiber organization. Both collagen and fibulin-1 are negatively associated with the change in FEV_1 reversibility. (J Allergy Clin Immunol 2023;■■■:■■■-■■■.)

Key words: *Airway remodeling, bronchial thermoplasty, extracellular matrix, severe asthma, collagen, fibronectin, FEV_1 reversibility*

Asthma is a highly prevalent pulmonary disease affecting more than 300 million patients worldwide.[1] Approximately 5% of all patients with asthma suffer from severe asthma.[2] Patients with severe asthma may benefit from bronchial thermoplasty (BT), a bronchoscopic treatment based on delivering radiofrequency energy to large- and medium-sized airways,[3] with acute effects that have been reported to extend to the untreated and smaller airways.[4,5] BT targets structural airway remodeling, the term describing the structural alteration of the airway wall, which is a prominent feature of asthma.[6,7] Airway remodeling involves an increase in airway smooth muscle (ASM) mass, thickening of the reticular basement membrane (RBM), and changes in extracellular matrix (ECM) proteins. RBM thickening and increased ASM mass are associated with loss of lung function,[8] and changes in several ECM proteins are associated with an increase in mucus secretion and bronchial hyperreactivity.[9-11] With the effect on airway remodeling, BT aims to improve asthma symptoms and quality of life and reduce exacerbations. Several randomized controlled trials have shown improvements in quality of life and clinical outcomes after BT treatment,[12-15] but how this relates to changes in the airway wall is largely unclear. Previous bronchial biopsy studies have focused on the impact of BT on ASM, with a more than 50% decrease in the ASM mass after BT being consistently reported, including the recently published TASMA trial.[16-22] However, in most of these studies, reductions in ASM mass failed to associate with improvements in asthma control and asthma-related quality of life after BT, except for the study by Ladjemi et al.[18] Some small studies have also examined the changes in collagen types[17,22] and RBM following BT,[16,18,21,23] of which 1 study found a correlation between the RBM thickness and asthma control test.[18] Chakir et al[17] reported reduced collagen type I within the basement membrane region 6 weeks after BT, but because of the small number of patients, no

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Abbreviations used
ACQ: Asthma Control Questionnaire
AQLQ: Asthma Quality of Life Questionnaire
ASM: Airway smooth muscle
BT: Bronchial thermoplasty
ECM: Extracellular matrix
RBM: Reticular basement membrane
TWOMBLI: The Workflow Of Matrix BioLogy Informatics

correlations between histopathologic findings and clinical parameters were detected. One study found an increased deposition of total collagen parallel with the reduction in ASM area,[16] but no correlations with clinical parameters considering this finding were reported.

Although reports about collagen deposition following BT have been observed in small patient cohorts, extensive analyses of changes in ECM components including elastin, fibulin-1, and fibronectin within the airway wall have not been examined after BT. Moreover, potential associations between changes in ECM composition and changes in clinical response and pulmonary function are unclear. Therefore, this study aimed to assess the effect of BT on different ECM components in the airway wall. In addition to the amount of ECM components present in the airway wall, the structural arrangement of these fibers dictates how cells respond to the microenvironment and contribute to the pathological process.[24,25] Therefore, we did evaluate the changes in RBM thickness and major ECM components (total collagen, elastin, fibronectin, and fibulin-1), together with the collagen fiber structures after BT. We hypothesized that histochemical alterations are associated with pulmonary function and treatment response.

METHODS

Subjects and study design

This study is a substudy of the TASMA trial (Clinical trials.gov NCT02225392) in which patients with severe asthma were treated with BT.[20] The study design of the TASMA study has been previously published.[20] Demographic data, including a bronchoscopy to obtain biopsies from (sub)segmental carinas, were collected at baseline. After baseline bronchoscopy, patients were randomized into an immediate BT treatment group and a 6-month delayed BT treatment group (control group), as previously described.[20] For the control group, additional visits were scheduled after 6 months of standard clinical care, including a research bronchoscopy with endobronchial biopsy sampling. In both groups, patients were followed up for 6 months after BT treatment, after which all patients underwent a post-BT bronchoscopy to obtain further biopsies.

Bronchial thermoplasty

Treatment procedures were performed according to current guidelines[3] and under conscious sedation (remifentanil/propofol)[26] or general anesthesia according to the current recommendations[3,27] using the Alair System (Boston Scientific, Marlborough, Mass). Patients were treated with prednisolone 50 mg starting 3 days before the procedure, on the day itself, and 1 day thereafter.

Clinical assessment

Clinical response to BT was measured by the 6-item Asthma Control Questionnaire (ACQ-6) and the Asthma Quality of Life Questionnaire (AQLQ) at baseline and 6 months after BT. FEV_1 reversibility (postsalbutamol FEV_1% predicted − presalbutamol FEV_1% predicted) and methacholine challenge (PC_{20}) test results were evaluated at baseline and 6 months after treatment.

Histology processing and analysis

Biopsies were paraffin-embedded and 5-μm sections were cut and mounted on microscope slides (3 sections per biopsy per slide). Sections were stained with hematoxylin and eosin to examine the RBM thickness, picrosirius red for total collagen, and antibodies specific for elastin, fibulin-1, and fibronectin to investigate ECM composition using standard protocols.[28-30] Sections were scanned using a Nanozoomer digital slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan). From each biopsy, 2 stained sections were included for analysis. Individual images were checked for quality by 4 independent reviewers and excluded when the RBM, lamina propria, ASM, or epithelium layer was missing or with extensive crushing artifacts. Digital analyses were performed to measure the RBM thickness and the percentage surface of tissue area positive in the whole biopsy for total collagen, elastin, fibulin-1, or fibronectin. The percentage of stained area was quantified using the FIJI ImageJ software (National Institutes of Health, Bethesda, Md). The ECM protein analyses used have been extensively described by Koloko Ngassie et al.[31] The Workflow Of Matrix BioLogy Informatics (TWOMBLI) plugin in ImageJ was used to investigate the collagen fiber structural characteristics.[32] Full details are provided in the Online Repository at www.jacionline.org. All stain analyses were blinded.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 28 (IBM, New York, NY). Demographic parameters were presented as mean ± SD or median with interquartile ranges. Not-normally distributed data were natural log– or log_{10}-transformed. A linear mixed-effects regression analysis with a random intercept on subject and biopsy was performed to identify significant differences before and after BT and differences in changes before and after standard clinical care and BT. Differences in changes in RBM and ECM parameters during standard clinical care compared with BT were statistically tested in the control group by including an interaction term between BT and change during BT in the linear mixed model ($n = 13$). To investigate whether the number of BT activations is associated with the changes in RBM and ECM parameters, we performed additional analyses. For this purpose, we included the number of activations, which was divided into high and low on the basis of the median number of activations. The effect of BT on clinical characteristics of patients in the total group was determined using paired *t* tests or the Wilcoxon signed-rank test. To analyze whether the change in histology was associated with the change in clinical parameters, a Pearson correlation analysis was performed on both histochemical and clinical parameter delta values (mean post-BT − mean pre-BT) and was tested for associations. Two-sided *P* values were used, with a statistical significance at P less than .05.

TABLE I. Baseline characteristics of patients with severe asthma

Characteristics	No. of patients (N = 30)
Sex (male/female)	7/23
Age (y)	47 ± 12
Age of asthma onset (y)	21 ± 18
BMI	28 ± 4
No. of patients with a history of smoking	10
Pack years	10 ± 8
Medication	
Dose of LABA (μg/d salmeterol equivalents)	140 ± 63
Dose of ICS (μg/d fluticasone equivalents)	1181 ± 616
No. of patients on maintenance use of OCS	8
Dose (mg/d)	12 ± 6
Asthma control	
Exacerbation rate/6 mo	2.23 (1.8-5.3)
ACQ-6 score	2.68 ± 0.68
AQLQ score	4.08 ± 0.94
Total serum IgE (kU/L)	164 (18.4-202)
Blood eosinophil count (10^9/L)	0.22 (0.07-0.30)
Lung function	
FENO	16.0 (12.6-33.6) (n = 24)
Pre-BD FEV_1 (% predicted)	86.10 ± 22.3
Post-BD FEV_1 (% predicted)	98.37 ± 19.8
FEV_1 reversibility (%)	13.67 ± 15.3
PC_{20} (mg/mL)	0.35 (0.03-2.98) (n = 29)
BT activations (total)	206 ± 55

Data are presented as mean ± SD or median (IQR). PC_{20} data were not available for 1 patient because of the inability to withhold asthma medications for the methacholine challenge test.

BD, Short-acting bronchodilation; *BMI*, body mass index; *FENO*, fraction of exhaled nitric oxide; *ICS*, inhaled corticosteroid; *IQR*, interquartile range; *LABA*, long-acting β-agonist; *OCS*, oral corticosteroid; *PC_{20}*, methacholine provocation test.

TABLE II. Clinical characteristics before and after BT

Characteristics	Mean ± SEM		Mean difference (95% CI)	*P* value
	Before BT	After BT		
ACQ-6 score	2.68 ± 0.12	2.22 ± 0.19	−0.40 (0.26 to 0.78)	.037*
AQLQ score	4.08 ± 0.17	4.77 ± 0.22	0.66 (−1.08 to −0.23)	.004*
Pre-BD FEV_1 (% predicted)	86.10 ± 4.08	89.81 ± 4.17	3.63 (−8.35 to 1.09)	.13
Post-BD FEV_1 (% predicted)	98.37 ± 3.64	97.31 ± 3.16	1.86 (−2.32 to 6.04)	.37
FEV_1 reversibility (%)	13.67 ± 2.94	7.04 ± 2.38	−6.63 (1.92 to 1.34)	.008*
PC_{20} (mg/mL)† (n = 25), median (IQR)	0.25 (0.03 to 2.42)	0.42 (0.05 to 3.28)	0.02 (−0.17 to 0.75)	.19
Exacerbations (per 6 mo)	2.23 ± 0.44	0.57 ± 0.18	1.67 (0.68 to 2.65)	.002*

Data are presented as mean ± SEM. Within-group analyses were performed using paired *t* test.

BD, Short-acting bronchodilation; *IQR*, interquartile range; *PC_{20}*, methacholine provocation test.

*Significant difference with $P < .05$.

†Values were log_{10}-transformed for statistical analysis. PC_{20} post-BT data were not available for 5 patients because of the inability to withhold asthma medications for the methacholine challenge test.

RESULTS

Subjects

Thirty patients with severe asthma from the TASMA trial[20] were included in this substudy to investigate the change in ECM components before and after BT in bronchial biopsies and its association with pulmonary function and treatment response. Baseline demographic data, asthma control scores, and pulmonary function measurements are provided in Table I.

Clinical characteristics

Clinical characteristics before and 6 months after BT and the changes therein are provided in Table II. The ACQ-6 score reduced by 0.4 after BT (mean score, 2.22) compared with that pre-BT (mean score, 2.62; $P = .037$). For the AQLQ score, a mean difference of 0.66 was observed after BT (mean score, 4.77) compared with pre-BT (mean score, 4.08; $P = .004$). FEV_1 reversibility reduced by 6.63% (post-BT mean, 7.04%; pre-BT mean, 13.67%; $P = .008$). The exacerbation rate reduced from 2.23 (pre-BT) to 0.57 6 months after BT (mean difference, 1.67; $P = .002$).

ECM components in the airway wall

Reticular basement membrane. RBM analysis was performed in 25 patients. A total of 197 biopsy sections (pre-BT, n = 98; post-BT, n = 99) were analyzed (Fig 1). BT decreased the maximum RBM thickness of 7.28 μm to 5.74 μm, with a mean change of 1.54 μm (95% CI, −2.94 to −0.13; $P = .03$; relative reduction of 21%) (Fig 2). In the control group

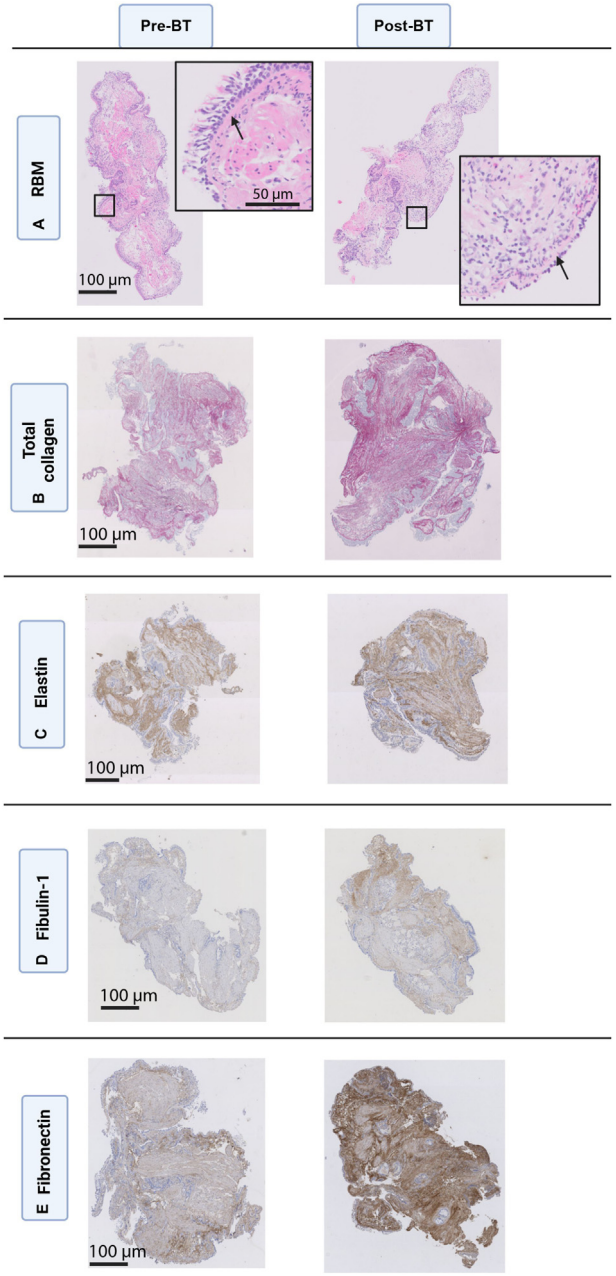

FIG 1. A-E, RBM thickness and ECM components of the airway wall before and after BT. Biopsies were analyzed with immunohistochemical stains before and after BT for RBM thickness (H&E-stained slides) (**A**) (the RBM is indicated by an arrow), total collagen (picrosirius red–stained slides) (**B**), elastin (**C**), fibulin-1 (**D**), and fibronectin (**E**). *H&E*, Hematoxylin and eosin.

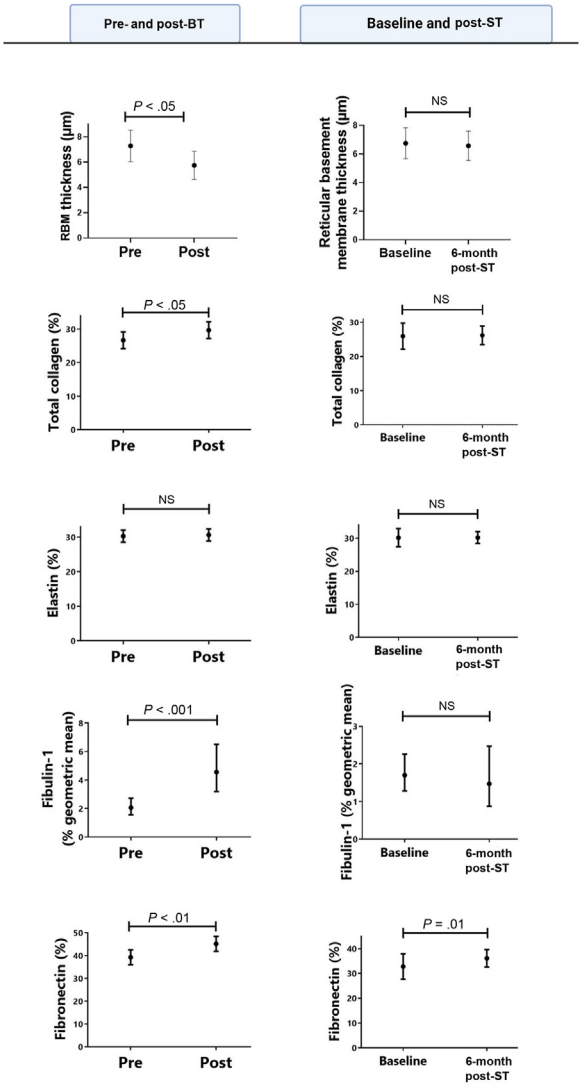

FIG 2. ECM components of the airway wall before and after BT and before and after standard therapy. *Left panel*, Differences before and after 6 months of BT. RBM thickness (H&E-stained slides): n = 25 patients; pre-BT, n = 98 biopsy sections; post-BT, n = 99 biopsy sections. Total collagen: n = 30 patients; pre-BT, n = 116 biopsy sections; post-BT, n = 115 biopsy sections. Elastin: n = 30 patients; pre-BT, n = 118 biopsy sections; post-BT, n = 115 biopsy sections. Fibulin-1: n = 30 patients; pre-BT, n = 119 biopsy sections; post-BT, n = 121 biopsy sections. Fibronectin: n = 30 patients; pre-BT, n =114 biopsy sections; post-BT, n = 114 biopsy sections. *Right panel*, Differences before and after 6 months of standard therapy. RBM thickness (H&E-stained slides): n = 8 patients; baseline, n = 36 biopsy sections; after ST, n = 36 biopsy sections. Total collagen: n = 13 patients; baseline, n = 47 biopsy sections; after ST, n = 46 biopsy sections. Elastin: n = 13 patients; baseline, n = 48 biopsy sections; after ST, n = 48 biopsy sections. Fibulin-1: n = 13 patients; baseline, n = 48 biopsy sections; after ST, n = 48 biopsy sections. Fibronectin: n = 13 patients, baseline, n = 44 biopsy sections; after ST, n = 47 biopsy sections. *H&E*, Hematoxylin and eosin; *NS*, not significant; *ST*, standard therapy.

(n = 8), no change in RBM thickness was found after 6 months of standard therapy (mean, 6.57 µm) compared with baseline (mean, 6.75 µm), with a mean difference of 0.17 µm (95% CI, −0.47 to 0.82; P = .59) (Fig 2). A reduction in RBM thickness was observed after BT when compared with standard therapy (P < .001; Table III). The number of BT activations did not associate with the change in RBM thickness (regression coefficient [B] = −1.15; 95% CI, −3.63 to 1.33; P = .36).

Total collagen. Total collagen analysis was performed in 30 patients. A total of 231 biopsy sections (pre-BT, n = 116; post-BT, n = 115) were analyzed (Fig 1). After BT, the surface area percentage of the biopsy tissue stained for collagen increased from 26.3% to 29.8% (relative increase of 13%; B = 3.42; 95% CI, 0.67 to 6.16; P = .02) (Fig 2). After standard therapy (n =13), collagen percentage surface area at baseline (mean, 24.7%) did not differ from the collagen area after 6 months (mean, 24.6%), with a mean difference of −0.08% (95% CI,

TABLE III. Changes in ECM components

	Change after standard therapy		Change after BT		Differences in changes between standard therapy and BT	
	B (95% CI)	*P* value	*B* (95% CI)	*P* value	*B* (95% CI)	*P* value
RBM (μm)	0.20 (−0.46 to 0.87)	.55	−1.70 (−2.31 to −1.08)	<.001	−1.90 (−2.81 to −1.00)	<.001
Collagen (%)	−0.03 (−4.47 to 4.41)	.99	6.47 (1.97 to 10.97)	.01	6.50 (0.18 to 12.82)	.04
Elastin (%)	−0.04 (−3.38 to 3.31)	.98	1.13 (−2.24 to 4.50)	.51	1.17 (−3.58 to 5.92)	.63
Ln(Fibulin-1) (%)	0.34 (−0.18 to 0.85)	.19	0.94 (0.43 to 1.46)	<.001	0.61 (−0.12 to 1.33)	.10
Fibronectin (%)	6.13 (0.34 to 11.93)	.04	2.73 (−3.03 to 8.49)	.35	−3.41 (−11.58 to 4.77)	.41

This table represents changes in RBM and other ECM components in the airway wall before and after standard therapy and BT and the differences in changes between these therapies (n = 13). The estimates of the change during standard therapy slightly differ from the estimates presented in the main text. This is because the analyses presented in the main text are stratified analyses based on treatment period, whereas in the current analyses, observations from both standard care and BT treatment are included in the same linear mixed model. The estimated covariances and random effects are based on all observations in the model.

−3.9 to 3.7; $P = .97$) (Fig 2). An increase in collagen area was observed after BT when compared with standard therapy ($P = .04$; Table III). A high number of activations was negatively associated with the change in total collagen area ($B = −13.0$; 95% CI, −17.92 to −8.12; $P < .001$). Patients with a high number of activations showed a higher total collagen area percentage at baseline ($B = 7.82$; 95% CI, 0.76 to 14.90; $P = .031$).

Elastin. Elastin analysis was performed in 30 patients. A total of 233 biopsy sections (pre-BT, n = 118; post-BT, n = 115) were analyzed (Fig 1). In the total group, no alterations were observed in the percentage surface area stained for elastin 6 months post-BT (mean, 30.6%) compared with pre-BT (mean, 30.3%), with a mean difference of 0.31% (relative change of 1%; 95% CI, −1.77 to 2.41; $P = .77$) (Fig 2). Similarly, in the control group, elastin tissue percentage surface area after 6 months of standard care (mean, 30.0%) did not differ from baseline (mean, 29.99%), with a mean difference of −0.041% (95% CI, −3.13 to 3.045; $P = .97$) (Fig 2). The change in elastin was not different after BT when compared with standard treatment (Table III). The number of BT activations did not associate with the change in the elastin area ($B = −2.89$; 95% CI, −7.07 to 1.28; $P = .17$).

Fibulin-1. Fibulin-1 analysis was performed in 30 patients, and a total of 240 biopsy sections (pre-BT, n = 119; post-BT, n = 121) were analyzed (Fig 1). BT increased the percentage surface area stained for fibulin-1 from 2.04% (geometric mean) to 4.56% (geometric mean) 6 months after BT (relative change of 124%; $B = 0.81$; 95% CI, 0.51 to 1.10; $P < .001$) (Fig 2). In the control group, no significant differences were observed in the amount of fibulin-1 that was present in the biopsy 6 months after standard therapy (geometric mean, 2.07%) compared with baseline (geometric mean, 1.47%) ($B = 0.34$; 95% CI, −0.21 to 0.89; $P = .2$) (Fig 2). The change in fibulin-1 was not different after BT when compared with standard treatment (Table III). The number of BT activations did not associate with the change in fibulin-1 area ($B = −0.5$; 95% CI, −1.12 to 0.05; $P = .08$).

Fibronectin. Fibronectin analysis was performed in 30 patients, and a total of 228 biopsy sections (pre-BT, n = 114; post-BT, n = 114) were analyzed (Fig 1). When examining fibronectin, an increase in the percentage surface area stained positive was observed 6 months post-BT (mean, 45.2%) compared with pre-BT (mean, 39.3%) (relative change of 15%; $B = 5.88$; 95% CI, 1.91 to 9.84; $P = .004$) (Fig 2). Strikingly, in the control group, an increase in fibronectin was also found 6 months after standard therapy (mean, 38.89%) compared with baseline (mean, 32.8%), with a mean difference of 6.14% (a relative change of 18%; $B = 6.14$; 95% CI, 0.53 to 11.76; $P = .03$) (Fig 2). The change in fibronectin was not different after BT compared with standard treatment (Table III). The number of BT activations did not associate with the change in fibronectin area ($B = 3.80$; 95% CI, −4.14 to 11.74; $P = .35$).

Total collagen fiber structure. The structural arrangements of the collagen fibers in the biopsies of 30 patients were analyzed before (116 biopsy sections) and after (115 biopsy sections) BT. A schematic overview of the measured collagen fiber structural parameters is shown in Fig 3, *A*. The average total length of the collagen fibers (Fig 3, *B*), the number of collagen fiber end points (representative of the number of individual fibers; Fig 3, *C*), the percentage of high-density matrix (Fig 3, *D*), and the curvature shape of the collagen fibers (Fig 3, *E* and *F*) were examined. There were no differences in the average total length of the collagen fibers and the number of collagen fiber end points between pre- and post-BT sections. A significant decrease in the percentage of high-density matrix was seen post-BT (mean, 0.86%) compared with pre-BT (mean, 0.87%; $P = .088$). An increase in the number of collagen fibers with a smaller curvature length scale (curvature mask, 30, indicating the number of collagen fibers with a low frequency of curves) was found post-BT (mean, 54.4) compared with pre-BT (mean, 53.0; $P = .01$), but no increase was found for the number of fibers that had a greater curvature length scale (curvature mask, 40, indicating the number of collagen fibers with a high peak amplitude of the curves).

Associations between changes in the ECM components and clinical outcomes after BT

A Pearson correlation coefficient was measured to assess potential associations between changes in ECM components and changes in clinical parameters (Table III). For this purpose, the change in each ECM parameter per patient was calculated (mean post-BT value − mean pre-BT value). Because no change in elastin was found after BT, this ECM protein was not included in the analysis. The clinical parameters that changed after BT were included in the analysis. No associations were found between the changes in the ECM components and the changes in ACQ-6 or AQLQ score. The changes in collagen and fibulin-1 were negatively associated with the change in FEV_1 reversibility (%) ($R = −0.47$; $P = .01$ and $R = −0.49$; $P = .05$, respectively) (Fig 4). This implicates that a higher increase in tissue area occupied by total collagen and fibulin-1 was associated with a larger reduction in FEV_1 reversibility. No association was found between the change in RBM thickness or fibronectin area change

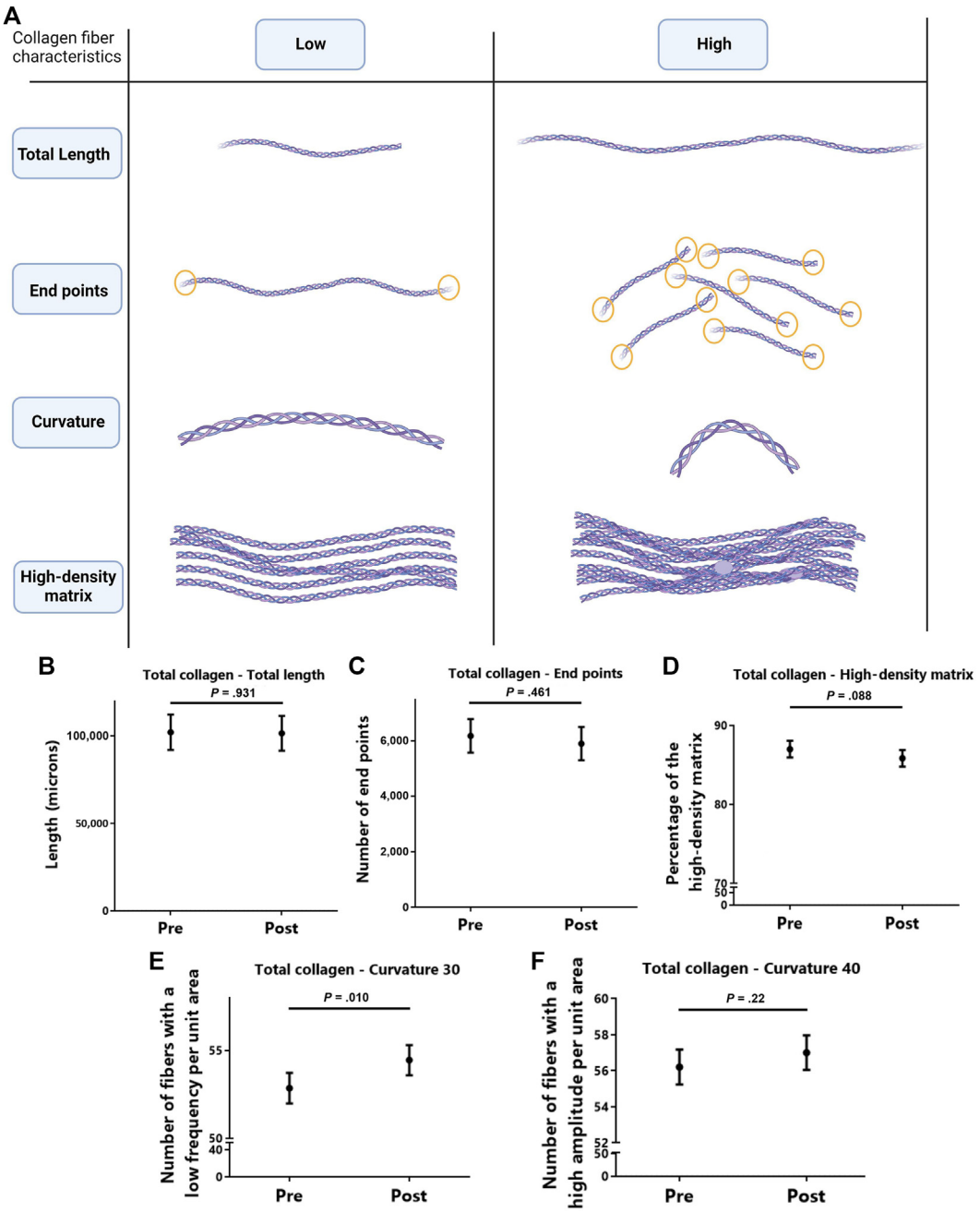

FIG 3. Collagen fiber structure characteristics pre- and post-BT. The collagen fiber structure before BT (n = 30 patients; 116 biopsy sections) and after BT (n = 30 patients; 115 biopsy sections) was analyzed using a linear mixed-effects regression analysis. For total length and end points, data were natural log–transformed to derive a normally distributed data set. **A**, A schematic overview of the parameters to determine collagen fiber characteristics using the TWOMBLI plugin in ImageJ.[26] End points are indicated by *yellow circles*. **B**, Total fiber length: 102216 pre-BT and 101638 post-BT. **C**, Number of collagen fiber end points: 6184 pre-BT and 5902 post-BT. **D**, Percentage of high-density matrix: 0.87% pre-BT and 0.86% post-BT. **E**, Number of collagen fibers with a low frequency of curves (curvature, 30): 52.9 pre-BT and 54.4 post-BT. **F**, Number of collagen fibers with a high peak amplitude of the curves (curvature, 40): 56.2 post-BT and 57.0 post-BT. The figure was created using BioRender (biorender.com).

and the change in FEV_1 reversibility (Table IV). No association was found between the changes in the ECM components and the clinical parameters at baseline (see Table E1 in this article's Online Repository at www.jacionline.org).

DISCUSSION

This is the first study to examine a comprehensive effect of BT on RBM thickness and different ECM components with its structural changes in the airway wall before and 6 months after

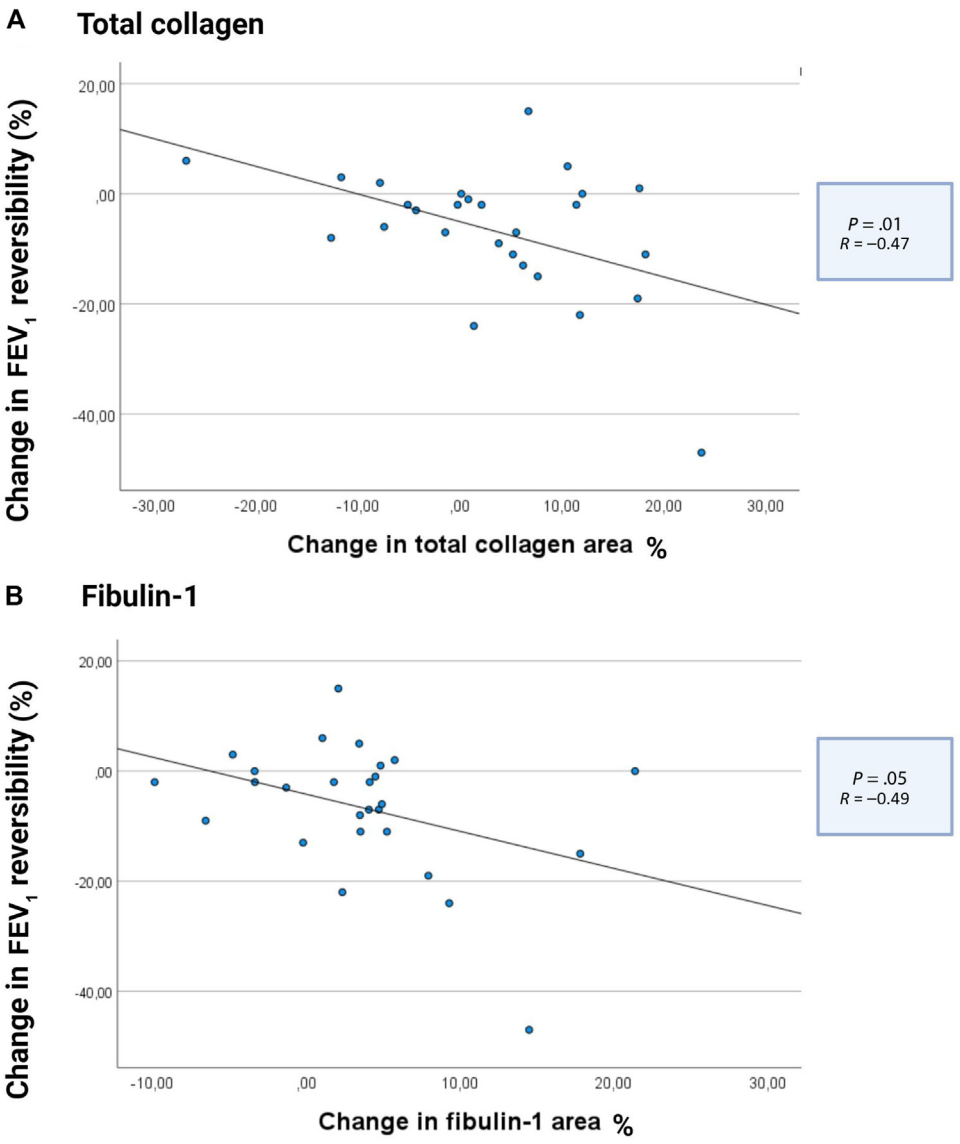

FIG 4. Associations between change in total collagen, fibulin-1 areas, and FEV_1% reversibility. Change was calculated by subtracting prevalues from postvalues. Associations were analyzed with Pearson correlation coefficient. **A**, Change in the percentage of biopsy that was positive for collagen and change in FEV_1 reversibility (%). **B**, Change in the percentage of biopsy that was positive for fibulin-1 and change in FEV_1 reversibility (%).

BT compared with 6 months of standard care. Furthermore, associations between these ECM changes and pulmonary function and treatment response were investigated. First, we found a decrease in RBM thickness and an increase in airway wall biopsy area occupied by total collagen, with a change in collagen fiber structure. Furthermore, increases in fibulin-1 and fibronectin were detected. A schematic overview of the structural changes found in this study is shown in Fig 5. A negative association was found between the changes in total collagen and fibulin-1 area and the change in FEV_1 reversibility after BT. These findings implicate that patients with a larger increase in total collagen and fibulin-1 area have a larger reduction in airway reversibility 6 months after BT, which suggests that reversibility is influenced by the ECM structure within the airway wall.

In the control BT treatment group, in which patients received standard care for 6 months, no changes were found in RBM thickness, total collagen, elastin, and fibulin-1 area at the end of this period (Fig 2). These data indicate that the standard asthma treatment had no effect on the ECM composition in the airway wall. This observation is in line with several previously published studies,[33-35] in which no changes in ECM composition were detected, following (anti-inflammatory) treatments including inhaled corticosteroids between 4 weeks and 10 years. Interestingly, unlike the other components, fibronectin did show an increase after standard therapy. This might reflect a repair response[36] because fibronectin deposition plays an important role in the pathogenesis of asthma by altering the extent of reepithelialization after airway injury as would be experienced in the

TABLE IV. Correlation between ECM changes and clinical parameters

	Δ Reversibility		Δ Exacerbation rate/6 mo		Δ ACQ		Δ AQLQ	
	R	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value
Δ RBM*	−0.01	.96	0.18	.39	−0.19	.35	−0.11	.60
Δ Total collagen	−0.47†	.01†	−0.26	.17	0.01	.96	−0.01	.92
Δ Fibulin-1	−0.49†	.05†	−0.09	.64	−0.15	.45	−0.10	.62
Δ Fibronectin	0.18	.37	0.03	.89	−0.11	.57	0.10	.61

Correlation between changes in RBM thickness, total collagen, fibulin-1, and fibronectin and clinical parameters. Associations were analyzed with Pearson correlation coefficient. The change was calculated by subtracting prevalues from postvalues.
*Data were available for 25 patients.
†Significant correlation with $P < .05$.

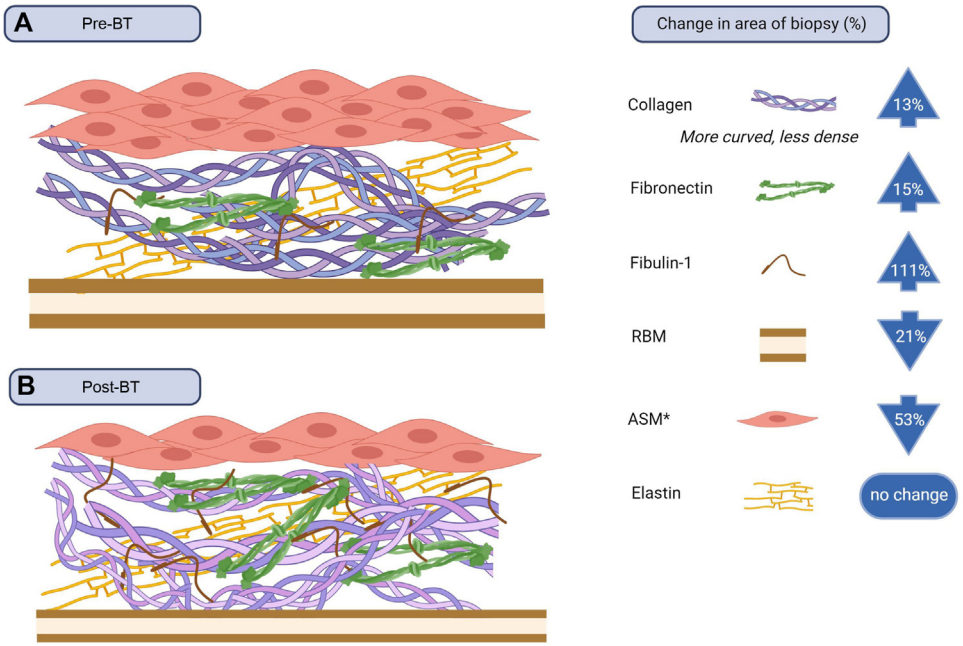

FIG 5. A schematic overview of airway remodeling changes after BT. **A**, The airway wall composition before BT. **B**, The airway wall composition 6 months after BT with a reduction in ASM, an increase in collagen where the structure of the fibers changed toward a less dense structure and with a larger angle of the curvature of the fibers, and an increase in fibulin-1 and fibronectin proteins. The figure was created using BioRender (biorender.com).

conducting airways after repeated asthma exacerbations. Both BT and exacerbations are an "injury" to the airways. Considering the high exacerbation rate of this study population, we postulated this might have contributed to fluctuations of fibronectin after standard therapy. However, after the correction for number of exacerbations, fibronectin was still significantly higher.

To further substantiate the measured ECM differences before and after BT, we tested the differences in effect between standard treatment and BT. For RBM and collagen, these differences between before and after standard care and BT were statistically significant; however, for the other ECM components, this could not be detected, probably because of the limited power of the study for this analysis.

The ECM in the airway wall is composed of a complex mixture of more than 100 proteins, glycoproteins, and other elements.[37] Given the impossibility of examining more than 100 proteins, on the basis of previous literature we selected those ECM proteins we considered to be significant proteins involved in the pathophysiology of asthma related to the impact of BT. Collagen and elastin are important macromolecules for contributing to the mechanical properties of the airway wall.[38] Fibulin-1 stabilizes ECM integrity through its interaction with other proteins, including collagen and fibronectin, an ECM adhesion protein.[39,40] Considering the role of these different ECM components in airway remodeling and asthma, these components were investigated. Moreover, these are the components known to be altered in asthma.[9] Interestingly, changes in elastin, fibulin-1, and fibronectin after BT have not been described earlier. Unlike total collagen, fibulin-1, and fibronectin, we did not find a change in elastin after BT. A possible explanation for this, in contrast to other ECM components, is that continuous remodeling of elastin does not occur throughout life, but rather only during the early years of life.[41] As such, elastin remodeling was less likely to be detectable. There is no consensus in the literature about elastic changes in asthmatic airways; both reduction and increase in elastic fibers have been reported.[42-44] The component with the relatively largest increase after BT was fibulin-1; however, the absolute surface percentage area occupied by fibulin-1 stays small

after BT. Fibulin-1 plays an important role in the restructuring and stabilization of the airway and is known to play a role in the regulation of tissue remodeling in respiratory diseases and is therefore elevated in asthma.[45] We suggest that the observed relatively large increase in tissue area occupied by fibulin-1 after BT might be reflective of its role in the stabilizing and restructuring of the ECM. Considering the role of ECM components in inflammation, such as modulation of immune cell recruitment, adhesion, migration, and the release of inflammatory mediators,[46] the changes in the different ECM components found in this study might have an impact on inflammation. It would be of great interest for future studies to examine whether the changes in the ECM after BT influence the inflammatory state of airways.

The effect of BT on different types of collagen has previously been investigated in 2 small studies with 9 patients in each study. Reduction of collagen types I and III has been described after 6 weeks and 12 months of BT treatment,[17,22] but in these studies only the airway wall areas directly under the RBM and submucosa were investigated, whereas in our study we examined the full airway biopsy area. In a gene expression study, Facciolongo et al[47] reported an increase in collagen type I gene expression in bronchial biopsies compared with baseline levels after a third BT session. In our study, the tissue area occupied by total collagen was investigated, instead of focusing on 1 specific collagen type. This is in line with a single report in 15 patients that measured an increase in total collagen by masson trichrome stain.[16]

A negative association between the high number of BT activations and the change in total collagen area before and after BT was found. To gain further insight, we analyzed whether the amount of collagen area at baseline was associated with the number of BT activations. It appears that the patients with more collagen area at baseline received a higher number of activations. A possible explanation for these findings might be that patients with more collagen at baseline have more extensively remodeled airways, which could make them less responsive to an increase in collagen after BT because of a stiffer matrix. However, these data should be interpreted with caution, because various patient-dependent factors may affect the number of activations provided during BT, including airway accessibility that might be related to airway size and/or inflammation.

The reduction of ASM mass after BT has been described in several studies.[18,19,48] Likewise, a more than 50% reduction of ASM mass has previously been reported in this study population.[20] The increase in the tissue area occupied by different ECM components after BT found in this study suggests that the ECM remodeling is acting to partly fill the void left by the reduction of ASM mass. Illustration of this correlation would be very complex because it is not a single protein that changes after BT, but the complete structure of the matrix in the airway wall.

In line with our results, a reduction in the RBM thickness following BT was described previously.[16,18,23] Two other reports could not detect a change in RBM thickness after BT, however, a different methodology was used (qualitative scale classification)[21] or performed in a very limited number of patients (n = 9).[22] In our control treatment group, no change in RBM thickness was evident after 6 months of standard treatment, which strengthens the observation that BT implicated the RBM thickness reduction in our study population.

In addition to the change in tissue area occupied by total collagen after BT, this study provides insight into the structural changes in the collagen fibers, an element of remodeling in the airway that has not been previously examined in BT studies. There was a trend toward a reduction in the percentage of high-density matrix present in the airway wall and an increase in the wave frequency of the curves (curvature 30). It appears that BT induces changes in the collagen fiber structure, following repair of the airway wall tissue after the treatment, which results in a less dense ECM meshwork. The increase in total collagen, in combination with the less dense structure, could support the association we found between the tissue area occupied by collagen and the reduction in FEV_1 reversibility. Considering the long-standing effect of BT,[49] it could be speculated that these changes persist even after 6 months; however, further long-term studies are needed. We postulate that BT induces remodeling of the airway wall, which results in a more stable ECM structure and therefore reduces airway wall reversibility in BT-treated patients with asthma. This extends the previous assumption that BT reduces airway hyperresponsiveness by decreasing the mass of the ASM layer with collagen production.[47] Previous trials explored the impact of BT on PC_{20} measurements.[12,13,20,50] In the first BT feasibility and AIR1 trial,[12,50] an improvement was found. However, other trials[13,20] did not find differences in PC_{20} measurements post-BT. The results of the first BT feasibility and AIR1 trial, together with our results that BT alters the ECM, imply that BT might have an impact on airway (hyper)responsiveness. Nonetheless, because of the mixed findings across studies, more research is needed.

One of the strengths of this study is the randomized design that enabled us to investigate the effect of both standard asthma treatment and BT on the ECM components in the airway wall with an integrated analysis of the RBM thickness. Second, the effect of BT on multiple ECM components and collagen fiber structure has been investigated, which, to our knowledge, has not been reported earlier. The investigation of the structural changes of the collagen fibers provides more insight into the nature of the airway remodeling alterations induced by BT. Lastly, our biopsies were taken throughout the entire bronchial tree to provide reflection of the total ECM content in the airways.

Several study limitations need to be acknowledged. At first glance, it appears that a relatively small number of patients were included. However, to our knowledge, this is the largest series that reports on comprehensive ECM changes after BT.[16,17,19,22] Second, although a control group of patients with severe asthma was included in the analysis, a healthy control group is not present in this study and therefore collagen structure and other ECM protein structural parameters in healthy airway walls are not well established. Finally, associations between histological airway remodeling features and clinical parameters might not have been detected because of the limited number of patients.

This study provides novel data and greater insight into the impact of BT airway remodeling. We showed that, after 6 months, BT results in a reduction in the RBM thickness and restructuring of the airway ECM, as demonstrated by an increase in both fibulin-1 and fibronectin, and an increase in the total collagen, with a resultant matrix structure that is less densely assembled. Changes in total collagen and fibulin-1 area were associated with a reduction in FEV_1 reversibility. Together with the decrease in ASM mass and the ECM changes that associate with reversibility, our data suggest that BT increases airway wall stability.

DISCLOSURE STATEMENT

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Clinical implications: BT induces ECM reorganization and airway wall stability.

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METHODS

Subjects and study design

Patients with severe asthma (18-65 years old) who fulfilled the World Health Organization[E1] and Innovative Medicine Initiative[E2] criteria were included after obtaining written informed consent. Patients were recruited in 2 centers in the Netherlands (Amsterdam University Medical Center and University Medical Center Groningen) and 2 centers in the United Kingdom (Royal Brompton Hospital and Chelsea & Westminster Hospital). This study was performed in compliance with the Declaration of Helsinki. Ethical approval was provided by the ethical committees of all the 4 centers.

BT and clinical procedures

Treatment procedures were performed according to current guidelines[E3] and under conscious sedation (remifentanil/propofol)[E4] or general anesthesia according to the current recommendations[E3,E5] using the Alair System (Boston Scientific). Patients were treated with prednisolone 50 mg starting 3 days before the procedure, on the day itself, and 1 day thereafter. Asthma medication remained unchanged during the study period.

Histology processing and immunohistochemistry

During bronchoscopy, endobronchial biopsies were obtained with large cup forceps. Biopsies were sliced into sequential serial sections (4 μm in thickness), which were numbered ascendingly. For representation of the full biopsy, we selected 2 sections for our analyses with at least a distance of 120 μm (30 sections) between them. Deparaffinized lung slices were incubated with citrate buffer (10 mM sodium citrate, pH 6), TRIS buffer (0.1 mol TRIS, pH 9), or TRIS-EDTA buffer (10 mM TRIS and 1 mM EDTA, pH 9) for 15 minutes at 100ºC for antigen retrieval for fibulin-1, elastin, and fibronectin, respectively. The slides were cooled for 30 minutes at room temperature and washed with PBS. These were then incubated with monoclonal anti–fibulin-1 antibody (1:75; Abcam, ab21536), polyclonal anti–elastin antibody (1:100; Cedarlane, CL55011AP), or polyclonal anti–fibronectin antibody (1:100; Abcam, ab6584) for 1 hour or overnight at 4ºC (fibulin-1 antibody), followed by anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary antibody (1:100; DAKO) at room temperature for 30 minutes. After washing, the slides were incubated with anti-rabbit or anti-goat horseradish peroxidase–conjugated tertiary antibody (1:100; DAKO). Diaminobenzidine (DAB; DAKO) was used as the chromogen and hematoxylin as the nuclear counterstain.

Sirius red

Deparaffinized lung slices were incubated with a 0.1% sirius red solution dissolved in aqueous saturated picric acid for 1 hour, washed in acidified water (0.5% acetic acid), dehydrated, and mounted with Permount Mounting Media (SP15-100; Fisher Chemical).

RBM and ECM image analysis

Images were captured using a Nanozoomer digital slide scanner (Hamamatsu Photonics). Slides were analyzed using image analysis software (ImageJ 1.53c, National Institutes of Health). RBM thickness was measured in hematoxylin and eosin–stained slides. In each biopsy, we manually delineated the RBM outer and inner borders along their lengths, up to 4 mm. Any histological artifacts or tangentially cut areas were avoided. A tool in Image-Pro Plus software performed automatic sequential measurements of the distance between the delineated outer and inner borders of RBM, presenting the average, minimum, and maximum distances as output expressed in micrometers.[E6] An example is given in Fig E1.

In each image, positive area (DAB and sirius red stain) and total tissue area were automatically measured. Total tissue was measured by converting the image to 8-bit type (gray). Pixels representing tissue were identified using the threshold feature of the ImageJ program. Positive area for the immunohistochemistry stains was identified by separation of the original images into blue (hematoxylin image) and brown (DAB image) using color deconvolution plugin by Landini.[E7] Color deconvolution involves isolation of the color information from histological red, green, and blue images containing multiple stains. To determine the correct optical density vectors for the red, green, and blue channel of hematoxylin, DAB, and sirius red, we followed the protocol as previously described by Ruifrok and Ruifrok.[E8] The vector used to isolate the colors for hematoxylin and DAB was 0.650,0.700,0.450,0.150,0.750,0.400,0.200,0.500,0.800 and that for sirius red was 0.650,0.700,0.450,0.150,0.750,0.400,0.200,0.500,0.800.

The pixel intensities of separated DAB/sirius red images range from 0 to 255. The value 0 represents the darkest shade of the color, whereas 255 represents the lightest shade of the color in the image. To assign an automated score, we categorized pixel intensity ranges as follows: 0 to 160, total staining; 0 to 53, strong staining; 54 to 107, medium staining, and 108 to 160, weak staining. Intensity values higher than 160 were set as background staining. A macro was developed to receive automated number of pixels in the different images. A score for expression in the images was calculated by dividing the positively stained area over the total tissue area.

The ECM protein percentage area analyses that were used have been described by Koloko Ngassie et al.[E9] The percentage of positive area was calculated by using the following formula, in which the percentage of positively stained tissue is calculated as follows:

$$\text{Area}\ (\%) = (\text{No. of pixels positive for NovaRed} / \text{No. of pixels in total tissue}) \times 100.$$

TWOMBLI analysis

Sirius red–stained sections were scanned with a Nanozoomer digital slide scanner (Hamamatsu Photonics) at 40× magnification. Images were resized to 25% and picrosirius red color was isolated from the images using a color deconvolution plugin.[E7] These images were analyzed with TWOMBLI, an ImageJ/Fiji[E10] plugin, to quantify patterns in ECM with respect to the total length of the fibers, end points, high-density matrix, and curvature, as described previously.[E11]

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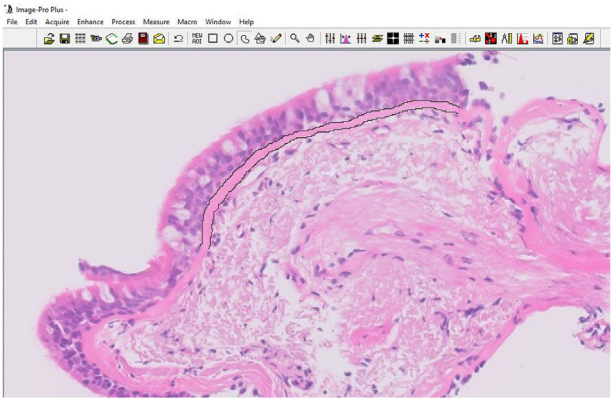

FIG E1. Exemplification of the RBM thickness assessment in an H&E-stained biopsy. *Black lines* are delineating the outer and inner borders of the RBM. *H&E*, Hematoxylin and eosin.

TABLE E1. Associations between ECM changes in clinical parameters at baseline

	Exacerbation rate/6 mo		ACQ		AQLQ		Pre-BD FEV_1		Post-BD FEV_1		Reversibility		PC_{20} (n = 25)*	
	R	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value	*R*	*P* value
RBM (n = 25)†	0.06	.79	0.08	.72	0.04	.86	−0.16	.49	−0.31	.14	−0.19	.40	0.10	.71
Total collagen	−0.34	.07	0.03	.90	−0.14	.45	0.10	.60	−0.04	.84	−0.20	.33	0.01	.98
Elastin	−0.14	.47	−0.05	.62	0.10	.62	−0.13	.50	−0.13	.49	0.02	.94	−0.12	.07
Fibulin-1	−0.18	.34	0.16	.40	0.08	.66	−0.08	.69	0.09	.62	0.27	.17	−0.14	.17
Fibronectin	0.23	.23	−0.05	.81	0.23	.22	−0.29	.13	−0.29	.12	0.15	.45	−0.12	.57

Associations between changes in RBM thickness, total collagen, fibulin-1, and fibronectin and clinical parameters at baseline. Statistical significance was tested with Pearson correlation coefficient.

BD, Short-acting bronchodilation; PC_{20}, methacholine provocation test.

*PC_{20} data were not available for 5 patients because of the inability to withhold asthma medications for the methacholine challenge test.

†RBM data were available for 25 patients.