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**Title: Airway smooth muscle reduction after Bronchial Thermoplasty in severe asthma correlates with FEV<sub>1</sub>**

**Short running title: Airway smooth muscle reduction after BT.**

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**To the Editor,**

Bronchial thermoplasty (BT) is a bronchoscopic treatment for severe asthma patients in whom the airways are treated with radio-frequency energy with the aim to improve asthma symptoms by reducing airway smooth muscle (ASM).<sup>1</sup> So far, three patient cohorts reported ASM mass reduction after BT by using  $\alpha$ -smooth muscle actin staining ( $\alpha$ -SMA).<sup>2-4</sup> However, the exact mechanism of action of BT and its related responder profile is unclear. Elucidating clinical parameters that predict response is a priority. Therefore, the objectives of this study are to: 1) assess the ASM in biopsies with two different staining methods; 2) detect the change in ASM mass after BT and compare this with the untreated right middle lobe (RML); 3) investigate if baseline ASM mass and ASM mass change correlates with baseline FEV<sub>1</sub>. Patients who fulfilled the World Health Organization or modified Innovative Medicines Initiative criteria of severe refractory asthma were included in the TASMA trial (Clin. Trials.gov NCT02225392).<sup>5,6</sup> Ethical approval was obtained (NL45394.018.13). After informed consent, clinical evaluation was performed and biopsies from (sub)segmental airway carinas obtained during bronchoscopy prior to BT. Bronchoscopy was repeated 6 months post-BT and airway biopsies were obtained from the BT-treated airways and the non-BT-treated RML.

Patients were treated with BT using the Alair system (Boston Scientific, Natick, USA).<sup>7</sup> Two desmin-stained (clone-33, biogenex, Fremont, USA) and  $\alpha$ -SMA (clone 1A4 DAKO, Santa Clara, USA) sections per biopsy of 2 biopsies per time-point and 1 biopsy of the RML were measured, blinded, by automatic digital image analysis (ImageJ, NIH, Bethesda, USA). ASM mass was defined as percentage (%) desmin or  $\alpha$ -SMA-positive area of the total biopsy area (Figure 1A).

Mann-Whitney test was performed for between group analysis and paired t-test or Wilcoxon signed-rank for paired analyses before and after BT (GraphPad Prism 5.01, San Diego, USA). Intraclass correlation coefficient (ICC) was calculated to assess the variability within biopsies and interpreted according to Landis-Koch: <0.2 poor, 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 substantial and 0.81-1 excellent.

18 severe asthma patients were evaluated in this ASM mass analysis before and after BT-treatment. Baseline characteristics are presented in Table 1.

Asthma Control Questionnaires (ACQ) and Asthma Quality of Life Questionnaires (AQLQ) significantly improved after BT (ACQ decreased from 2.6 ( $\pm 0.7$ ) to 1.9 ( $\pm 1.0$ ) ( $p=0.02$ ); AQLQ improved from 4.5 ( $\pm 0.95$ ) to 5.2 ( $\pm 0.99$ ) ( $p=0.01$ )). Additionally, exacerbation rates were significantly lower after 6 months showing a reduction from 1.75 (0.88;3.5 IQR) per 6 months before BT to 0.00 (0.00;0.25 IQR) per 6 months after BT ( $p=0.0034$ ). Medication use after BT was not modified in this study because asthma medication needed to be stable during the 6 months of follow up.

Desmin staining analyses showed >50% reduction of the median ASM mass after BT from 11.1% (5.5; 15.3 IQR) to 5.4% (2.6; 8.4 IQR) ( $n=18$ ;  $p=0.003$ ) (Figure 1B). The untreated RML remained unchanged at 6 months (median 10.7% (5.4; 14.8 IQR) to 9.2%

(3.4; 10.7 IQR) ( $n=17$ ;  $p=0.45$ ). Pre- and post-bronchodilator FEV<sub>1</sub> negatively correlated with baseline ASM mass ( $R=-0.63$ ;  $p=0.005$  and  $R=-0.63$ ;  $p=0.005$  respectively).

Furthermore, ASM mass decrease after BT negatively correlated with pre- and post-bronchodilator FEV<sub>1</sub> ( $R=-0.52$ ;  $p=0.025$  and  $R=-0.49$ ;  $p=0.04$ , respectively) (Figure 1C), indicating that patients with a lower FEV<sub>1</sub> had more ASM mass reduction after BT treatment. Additionally, a significant difference in ASM mass decrease was observed between patients with a FEV<sub>1</sub> below 80% as compared to patients with a FEV<sub>1</sub> larger than 80% (FEV<sub>1</sub><80% ( $n=7$ ): 8.8% (6.0; 9.7 IQR) vs. FEV<sub>1</sub>>80% ( $n=11$ ): 2.2% (0.3; 6.3 IQR;  $p=0.03$ ) (Figure 1D).

The same analyses were performed with  $\alpha$ -SMA staining which confirmed an ASM mass reduction after BT, although less profound (median 22.0% (17.6; 29.7 IQR) to 13.6% (9.5; 15.1 IQR) ( $n=18$ ;  $p=0.0005$ )). The ASM mass of the RML remained unchanged (baseline median ASM mass of 21.0% (17.5; 28.1 IQR) vs 18.8% (13.7; 25.1 IQR) at 6 months ( $n=17$ ;  $p=0.09$ )). The correlation with FEV<sub>1</sub> and baseline ASM mass or ASM mass decrease was not found with  $\alpha$ -SMA staining. A moderate ICC between biopsies of 0.52 for desmin and a fair ICC of 0.33 for  $\alpha$ -SMA was found.

This study demonstrates that BT treatment results in ASM mass decrease by two different staining methods and shows for the first time that baseline FEV<sub>1</sub> is negatively correlated with ASM mass decrease after BT. This might imply that BT is more effective in reducing ASM mass in asthma patients with airway remodelling. Previously, Benayoun et al. showed that in asthma patients, an increased ASM mass correlates with a decreased FEV<sub>1</sub>.<sup>8</sup> Furthermore, Chakir et al and Pretolani et al have demonstrated ASM reduction following BT using  $\alpha$ -SMA but none of these studies have shown a correlation between ASM mass and functional parameters.<sup>2,4</sup> In line with the study of Chakir et al. no correlations were found between clinical improvements and ASM.<sup>2</sup> A larger sample size is needed to explore these correlations. Since ASM is considered a key feature of airway remodelling, the correlations

found between FEV<sub>1</sub> and baseline ASM mass and ASM mass decrease after BT using desmin staining, supports the hypothesis that BT has an impact on airway remodelling.

The ASM mass of the RML did not significantly change after BT in this study, however a trend was seen towards reduction in the  $\alpha$ -SMA stained biopsies. One study showed a significant decrease in  $\alpha$ -SMA stained ASM mass in the RML after BT<sup>9</sup>, but this decrease was not confirmed in the larger follow-up study.<sup>4</sup> Due to the historical fear of atelectasis and RML syndrome, current recommendations do not advise to treat the RML.<sup>10</sup> A future trial investigating the safety and efficacy of reducing the ASM mass in the RML with BT is indicated.

We compared airway biopsies from different predefined (sub)segmental airway carinas which could potentially bias the results. However, comparing ASM biopsies from different areas has been shown feasible with a 7-12% coefficient of variation for the ASM mass between 10 biopsies taken from the upper lobes, lower lobes and RML within a patient.<sup>4</sup> Furthermore, we compared 2 different ASM staining methods, desmin and  $\alpha$ -SMA. The present data show and confirm that desmin staining specifically identifies the differentiated contractile ASM present in the ASM layer, whereas the  $\alpha$ -SMA also stains mucosal myofibroblasts, myoepithelial cells around glands and pericytes in capillaries/myofibroblast in blood vessels (Figure 1A).<sup>11,12</sup> We hypothesize that the correlation between ASM and FEV<sub>1</sub> depends on the fully differentiated contractile ASM located in the airway wall only and is not related to the  $\alpha$ -SMA stained glandular myoepithelial cells and vascular pericytes/myofibroblasts. This probably explains the higher pre-BT ASM mass for  $\alpha$ -SMA staining when compared to desmin staining, the less profound decrease of ASM mass and the absence of a correlation between ASM mass (change) and FEV<sub>1</sub>. In our opinion desmin is the staining method of choice for analyzing BT treatment effects on ASM.

In conclusion, ASM mass significantly decreased

6 months following BT treatment. Severe asthma patients with a  $FEV_1 < 80\%$  have a greater ASM mass at baseline and show the most reduction of ASM mass after BT as shown by ASM layer specific desmin staining. Whether this specific phenotype of severe asthma patients with low  $FEV_1$  responds best to BT, and whether a low  $FEV_1$  should therefore be a pre-requisite for BT, needs to be determined in a larger sample size.

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## Figure legends

### Figure 1.

Figure 1A. Desmin- and  $\alpha$ -smooth muscle actin-( $\alpha$ -SMA) stained airway biopsy sections before and after bronchial thermoplasty (BT) with the corresponding calculated airway smooth muscle (ASM) mass (%), showing ASM in the ASM layer (^), and for  $\alpha$ -SMA the staining of myoepithelial cells in mucus glands (\*) and pericytes in capillaries (#). Nuclei stained in blue (hematoxylin); ASM stained in brown (3'-Diaminobenzidine (DAB)).

Figure 1B. ASM mass reduction before and 6 months after BT assessed with desmin staining; median ASM mass (%) reduced from 11.1% (5.5; 15.3 IQR) before BT to 5.4% (2.6; 8.4 IQR) after BT (n=18,  $p=0.003$ ).

Figure 1C. Negative correlation between pre-BD- $FEV_1$  (%) and the ASM mass decrease showing that patients with a lower pre-BD- $FEV_1$  have more reduction of ASM mass 6 months after BT treatment (n=18; R= -0.52;  $p=0.025$ ). ASM mass decrease defined as pre-minus post-ASM mass (%) assessed with desmin. BD = bronchodilator.

Figure 1D. Difference in ASM mass decrease between BT-treated patients with a pre-BD- $FEV_1 < 80\%$  and pre-BD- $FEV_1 > 80\%$ . ASM mass decrease defined as pre-BT minus post-BT-ASM mass (%) assessed with desmin.  $FEV_1 < 80\%$  (n=7): 8.8% (6.0; 9.7 IQR) vs  $FEV_1 > 80\%$  (n=11): 2.2% (0.3; 6.3 IQR) ( $p=0.03$ ).

**Table 1. Baseline characteristics**

| Characteristics                                    | Baseline                |
|----------------------------------------------------|-------------------------|
| No. of patients                                    | 18                      |
| Sex (males/females)                                | 4/14                    |
| Age (y)                                            | 45 ( $\pm$ 13)          |
| Age of asthma onset (y)                            | 18 ( $\pm$ 16)          |
| Total serum IgE (kU/L)                             | 61.6 (17.05; 212.5 IQR) |
| No. of patients with positive blood allergy test   | 12                      |
| Pre short-acting BD $FEV_1$ (% predicted)*         | 89 ( $\pm$ 25)          |
| Post short-acting BD $FEV_1$ (% predicted)         | 101 ( $\pm$ 21)         |
| PC20 methacholine test (mg/ml) (n=17) <sup>#</sup> | 0.35 (0.03; 1.78 IQR)   |
| Blood eosinophil count ( $10^9/L$ )                | 0.15 (0.10; 0.29 IQR)   |
| ACQ score                                          | 2.6 ( $\pm$ 0.7)        |
| AQLQ score                                         | 4.5 ( $\pm$ 0.95)       |
| Dose of LABA ( $\mu g/d$ salmeterol equivalents)   | 147 ( $\pm$ 62)         |
| Dose of ICS ( $\mu g/d$ fluticasone equivalents)   | 1208 ( $\pm$ 495)       |
| No. of patients on maintenance use of OCS          | 6                       |
| Dose of oral prednisone (mg/d)                     | 12 ( $\pm$ 6)           |

|                                                   |                       |
|---------------------------------------------------|-----------------------|
| No. of patients on omalizumab                     | 2                     |
| ASM mass (%) assessed with desmin staining        | 11.1 (5.5; 15.3 IQR)  |
| ASM mass (%) assessed with $\alpha$ -SMA staining | 22.0 (17.6; 29.7 IQR) |

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Data are presented as numbers, mean ( $\pm$  SD) or median (IQR)

BD, bronchodilator; FEV<sub>1</sub>, forced expiratory volume in 1 second; LABA, long acting beta-2-agonist; ICS, inhaled corticosteroids; OCS, oral corticosteroids; ACQ, asthma control questionnaire; AQLQ, asthma quality of life questionnaire; ASM, airway smooth muscle;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

\*Short acting beta agonists were stopped at least 6 hours before the pulmonary function tests (long acting beta-agonists and long acting muscarinic antagonists continued).

<sup>#</sup>A single patient fulfilled the reversibility criteria of asthma, but tested negative on PC20 methacholine test and therefore was excluded from this analysis.

