Long-term modulation of airway remodelling in severe asthma following bronchial thermoplasty

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This study demonstrates that bronchial thermoplasty reduces smooth muscle and neural innervation of the airway up to 12 months post-therapy, whereas the airway epithelium is relatively resistant to thermal damage https://bit.ly/3wlQOJX

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Abstract

Rationale Bronchial thermoplasty is a mechanical therapeutic intervention that has been advocated as an effective treatment option for severe asthma. The mechanism is promoted as being related to the attenuation of airway smooth muscle which has been shown to occur in the short-term. However, long-term studies of the effects of bronchial thermoplasty on airway remodelling are few, with only limited assessment of airway remodelling indices.

Objectives To evaluate the effect of bronchial thermoplasty on 1) airway epithelial and smooth muscle cells in culture and 2) airway remodelling in patients with severe asthma who have been prescribed bronchial thermoplasty up to 12 months post-treatment.

Methods The distribution of heat within the airway by bronchial thermoplasty was assessed in a porcine model. Culture of human airway smooth muscle cells and bronchial epithelial cells evaluated the impact of thermal injury. Histological evaluation and morphometric assessment were performed on bronchial biopsies obtained from severe asthma patients at baseline, 6 weeks and 12 months following bronchial thermoplasty.

Results Bronchial thermoplasty resulted in heterogeneous heating of the airway wall. Airway smooth muscle cell cultures sustained thermal injury, whilst bronchial epithelial cells were relatively resistant to heat. Airway smooth muscle and neural bundles were significantly reduced at 6 weeks and 12 months post-treatment. At 6 weeks post-treatment, submucosal collagen was reduced and vessel density increased, with both indices returning to baseline at 12 months. Goblet cell numbers, submucosal gland area and sub-basement membrane thickness were not significantly altered at any time point examined.

Conclusions Bronchial thermoplasty primarily affects airway smooth muscle and nerves with the effects still present at 12 months post-treatment.

Introduction

Although patients with severe asthma comprise only 5–10% of the total asthma population, they account for a disproportionate amount of healthcare expenditure, owing to relatively frequent asthma exacerbations and lung function decline [1, 2]. Characteristic structural changes in the airway wall, termed airway remodelling, contribute to airway hyperresponsiveness (AHR) and exacerbations [3–5] and are relatively resistant to present treatment strategies [6]. The changes associated with airway remodelling in the large airways include

goblet cell hyperplasia, increased deposition of extracellular matrix proteins and collagen, sub-basement membrane thickening, myofibroblast infiltration, airway smooth muscle (ASM) hyperplasia and/or hypertrophy, increased angiogenesis and increased neural innervation [5, 7, 8]. Compared to patients with mild–moderate asthma, those with severe asthma have increased myofibroblast accumulation, subepithelial fibrosis, submucosal collagen and increased area of smooth muscle and submucosal glands [3, 4].

Bronchial thermoplasty is a mechanical therapeutic intervention approved for the treatment of patients with moderate or severe asthma [9]. Bronchial thermoplasty generates radiofrequency energy, which produces a transient increase in temperature within the airway wall. Although several studies have confirmed a reduction in ASM 6 months [10–15] and up to 2 years post-bronchial thermoplasty [16], the underlying mechanism of action is not clear [17, 18]. It is hypothesised that reduced ASM results in a decreased ability of airways to constrict, and a subsequent decrease in airway hyperresponsiveness, which in turn has been correlated with improved asthma control [10, 13], reduced frequency of asthmatic exacerbations and hospitalisations [19–21]. Recent studies describe reduced neural innervation post-bronchial thermoplasty [13, 22]. Studies have reported reduced collagen within the sub-basement membrane (the prominent eosinophilic hyaline layer beneath the true epithelial basement membrane, which is characteristic of asthma) 6 weeks after bronchial thermoplasty [11] and increased collagen in this layer 3 months following bronchial thermoplasty [10]. However, the effect of bronchial thermoplasty on collagen deposition within the submucosa itself has not been studied. Since there is some concern that bronchial thermoplasty may trigger compensatory remodelling and scarring, our aim was to do a comprehensive assessment of airway remodelling up to 1 year post-treatment.

The hyperthermia produced by bronchial thermoplasty affects the fluidity and stability of cellular membranes and impairs the function of transmembrane transport proteins and surface extracellular surface receptors [23, 24], which are susceptible to further apoptotic and/or necrotic signalling as proteins involved in DNA replication and stability are damaged [17]. However, studies examining the heat profile and short-term effects of bronchial thermoplasty are few and incomplete [17, 18], and few safety data associated with the thermal profile of the device are available. Therefore, we initially assessed the temperature profile of the bronchial thermoplasty device in an animal model and then went on to replicate similar conditions in cell culture. Finally, we evaluated the effect of bronchial thermoplasty on indices of airway remodelling in a cohort of well-characterised patients with severe asthma who were prescribed bronchial thermoplasty, over a 12-month period.

Methods

See the supplementary material for detailed methods.

Animal studies

To determine the thermal profile of bronchial thermoplasty in the airway wall, a 6-month-old (~40 kg) White-Landrace piglet bronchiole, with relatively similar airway anatomy to the adult human lung, was used [25]. The piglet was treated in compliance with the Canadian Council for Animal Care and the protocol received approval from the University of Calgary animal care and use committee (AC14-0135). The thermal profile of bronchial thermoplasty was captured using a thermal camera (FLIR SC660; Wilsonville, OR, USA) in 3–5-mm diameter bronchioles in three different lobes. The temperature was recorded every 1.0 ms starting immediately before activation of the bronchial thermoplasty instrument, the length of the activation time (10 s) and 8 s after the bronchial thermoplasty instrument shut off. The results were used to inform the cell culture temperature profiles.

Cell culture studies

Primary human ASM and bronchial epithelial (HBE) cells were obtained [26]. Cells were treated with media heated to 37°C (control), 65°C or 85°C for 10 s and cultured further for 24 h at 37°C. ASM cell viability was determined 0.5, 3 and 24 h post-treatment.

Study participants

Participants (aged 18–65 years) diagnosed with asthma and prescribed regular maintenance inhaled corticosteroid (>1000 mg·day^{-1} beclomethasone or equivalent) and long-acting β-agonist (>100 µg·day^{-1} salmeterol or equivalent) were recruited from the investigators' tertiary-care asthma clinics for observational study (table 1). The University of Calgary conjoint health research ethics board approved the protocol (REB14-1100). All participants provided written informed consent prior to study participation. Following baseline bronchoscopy and biopsies, participants underwent bronchial thermoplasty (Alair; Boston Scientific, Natick, MA, USA) and were subsequently assessed at 1, 3, 6, 9 and 12 months

TABLE 1 Patient characteristics at intake

Male/female	6/3
Age at intake (years)	50±2
Asthma onset (years)	23±7 (1–46)
BMI (kg·m^{-2})	28.3±1.8
Systolic BP (mmHg)	124±4
Diastolic BP (mmHg)	81±3
Pre-bronchodilator FEV_1 (L)	2.3±0.2
FEV_1 (% pred)	70.8±2.9
Pre-bronchodilator FVC (L)	4.1±0.3
FVC (% pred)	101.8±2.3
ACQ	2.1±0.4
AQLQ	4.8±0.6
Medications	
Corticosteroids	
Budesonide/formoterol	6
Fluticasone/salmeterol	1
β-agonists	
Salbutamol	9
Terbutaline	1
Leukotriene inhibitors	
Montelukast	4
IgE monoclonal antibodies	
Omalizumab	2
Muscarinic receptor antagonists	
Tiotropium	2
Glycopyrronium	1
Comorbidities	
Sinusitis	1
Eczema	3
Hypertension	2
ASA allergy	1
Post-nasal drip	2
Nasal polyps	1
Depression	1
Psoriasis	1

Data are presented as n or mean±SEM (range), unless otherwise stated. BMI: body mass index; BP: blood pressure; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ: Asthma Control Questionnaire; AQLQ: Asthma Quality-of-Life Questionnaire; Ig: immunoglobulin; ASA: acetylsalicylic acid.

post-procedure. Repeat bronchoscopy and biopsies were performed at 6 weeks and 12 months (figure 1). Nine participants completed the study.

Histology and morphometric analyses

Routine histological examination using haematoxylin and eosin (H&E) was performed, with additional stains used to identify goblet cells (periodic acid schiff) and collagen (picrosirius red (PSR) with the aid of polarised light). Goblet cells were normalised to the sub-basement membrane length. The thickness of the sub-basement membrane area, the prominent eosinophilic hyaline layer beneath the true epithelial basement membrane (also variably referred to as the "reticular basement membrane"), was measured by multiple point counts [27]. It consists of multiple components other than just collagen, such as matrix proteins [28–32]. The submucosal collagen deposition was analysed using the natural birefringent properties of collagen when stained with PSR, normalised to the total submucosal area of the biopsy. This method is regarded as highly sensitive in the assessment of collagen [33, 34]. Immunohistochemistry using antibodies for α-smooth muscle actin (SMA), CD31 and S100 was used to identify smooth muscle, vascular endothelial cells and nerves, respectively. Sections stained with H&E, α-SMA and CD31 were assessed using stereology (Stereo Investigator; MBF Bioscience, Williston, VT, USA). ASM, neural bundles, submucosal glands and submucosal collagen were normalised to submucosal area; the percentage area of red and green birefringent collagen, representative of type I and III collagen, respectively, was normalised to total collagen area. Investigators performing the analysis were blinded to patients and biopsy schedules, and a subset of biopsies were recounted to ensure repeatability.

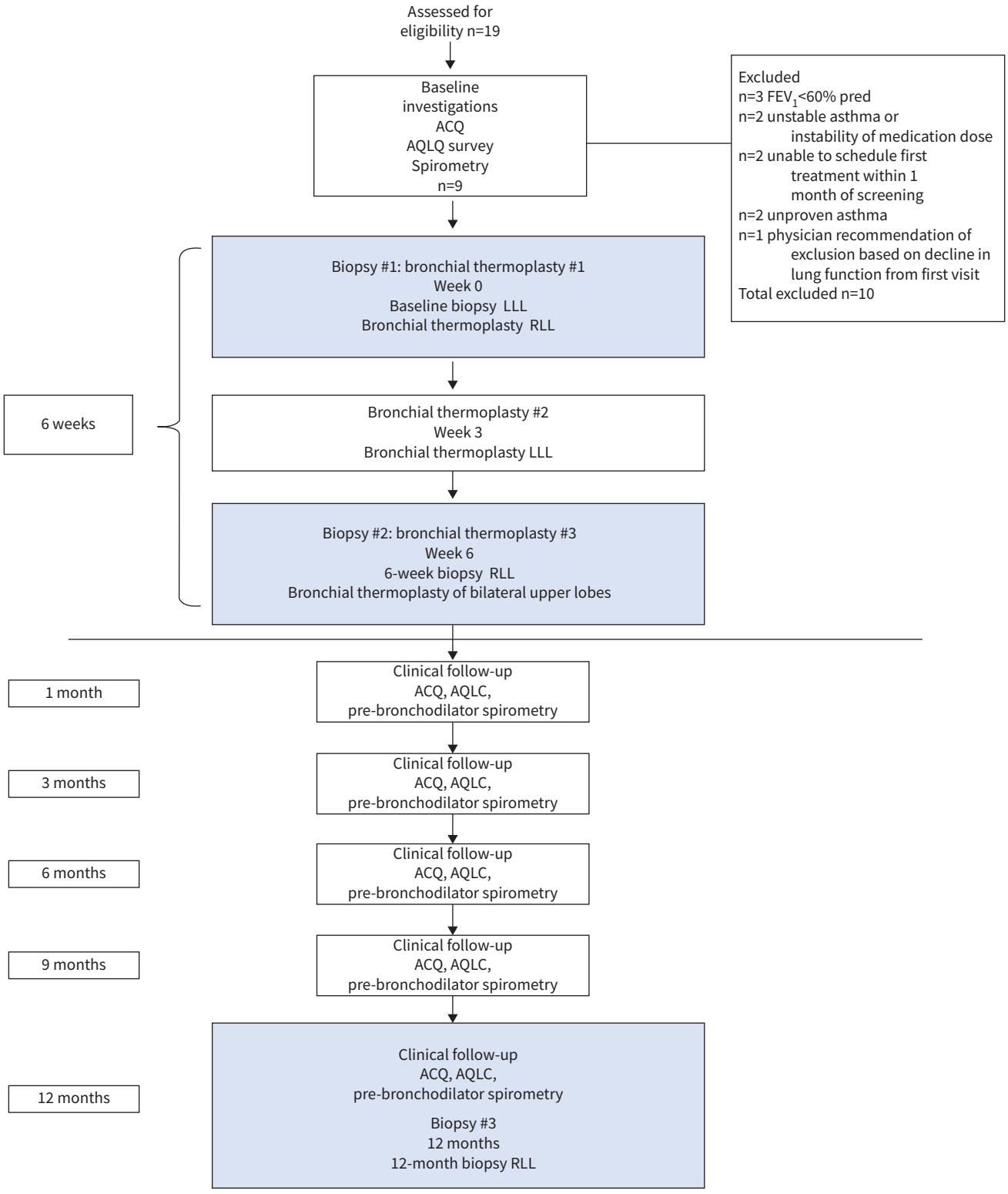

FIGURE 1 Study design. Flow chart outlining the stages of the study and participant inclusion and exclusion. Patients underwent four endobronchial biopsies over the course of 12 months. The first biopsy (#1) was from the left lower lobe (LLL) (baseline) immediately before the first bronchial thermoplasty treatment to the right lower lobe (RLL). Bronchial thermoplasty (#2) to the LLL occurred at week 3. At 6 weeks, the second biopsy (#2), which was from the RLL, occurred immediately before the third bronchial thermoplasty (#3) which was applied to the right and left upper lobes. Clinical follow up occurred at 1, 3, 6, 9 and 12 months. The last biopsy was at 12 months, (#3) from the RLL. ACQ: Asthma Control Questionnaire; AQLQ: Asthma Quality-of-Life Questionnaire; FEV_1: forced expiratory volume in 1 s.

Statistical analysis

All data were normally distributed, as assessed by the Shapiro–Wilks test, with equal variance, as tested by the Brown–Forsythe test. All data are expressed as mean±SEM or 95% CI. Data were analysed by two-tailed mixed model repeated-measures ANOVA with the Student–Newman–Keul *post hoc* analysis, paired t-test, Chi-squared test or Spearman correlation using Sigmaplot 14.0 (Systat, San Jose, CA, USA) for analysis and plotted in GraphPad Prism 6.0. Significance was set *a priori* at $p<0.05$.

Results

Thermal profile of the bronchial thermoplasty procedure

Thermal mapping of the effect of bronchial thermoplasty in the piglet bronchiole was performed using a thermal camera (figure 2a,b and supplementary video), replicated three times in three bronchioles. A maximal temperature of 82.9±8.3°C was reached ~7 s after initiating bronchial thermoplasty at the four points of contact. At these contact points, the average temperature approximated 64°C during 18 s of recording, decreasing to ~50°C at the end. The airway between the points of contact did not reach this maximal temperature, but increased to a maximum of ~60°C and then decreased to ~55°C during 18 s of recording.

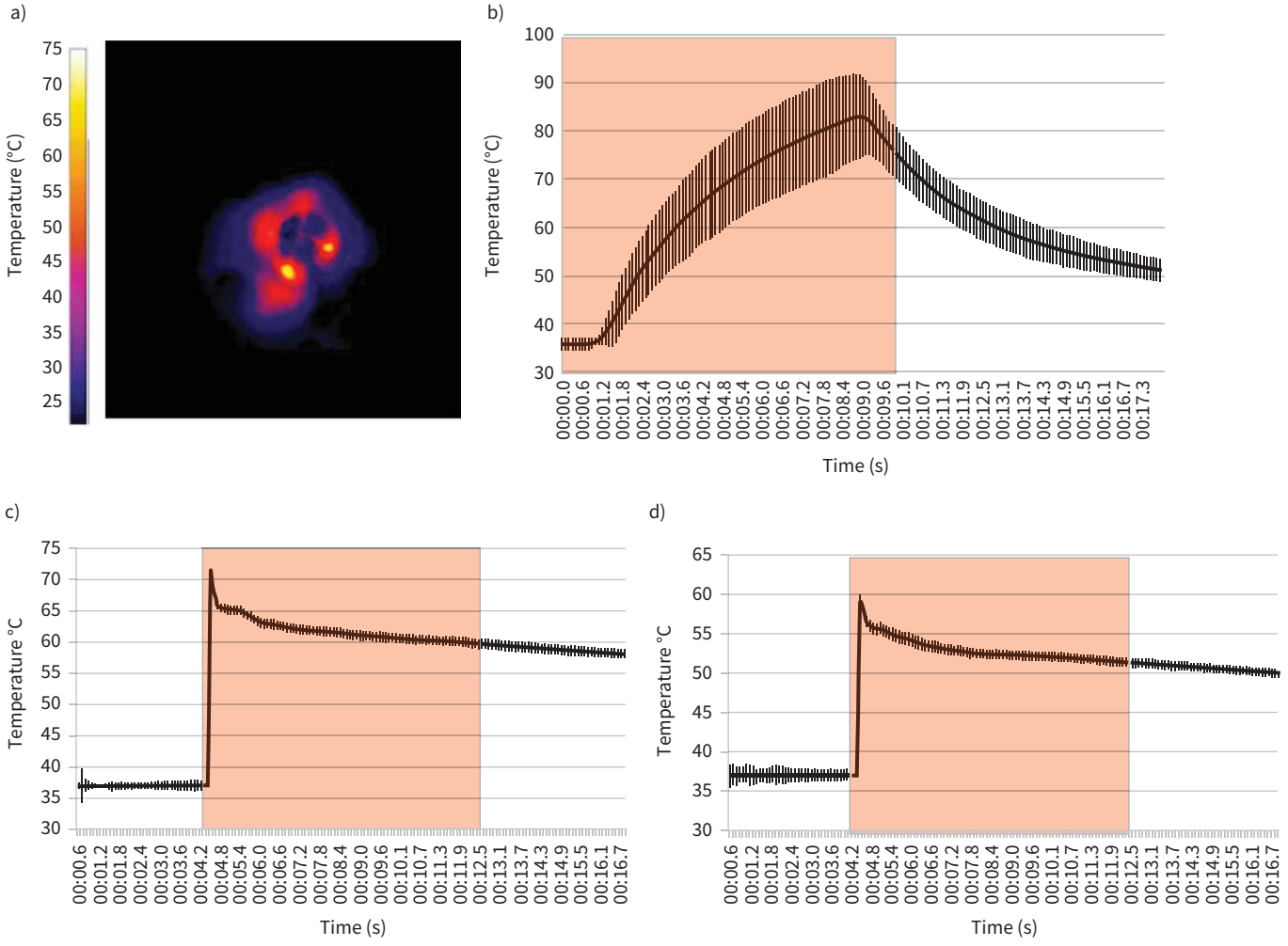

FIGURE 2 Thermal camera heat profiles. a) A representative frame from the thermal video demonstrating heat distribution during the bronchial thermoplasty procedure. The wire array catheter is seen within the airway of an excised porcine lung. The increase in temperature is localised to the points of contact between the expandable wires of the catheter and the airway wall. b) The heat distribution within the porcine airway during bronchial thermoplasty activation over 18 s; average of three activations. c) Representative thermal profile of a cell chamber, initially at a temperature of 37°C, after the addition of 85°C media. Average temperature of 61°C over 18 s, "maximal temperature profile". d) Representative thermal profile of a cell chamber, initially at a temperature of 37°C, after the addition of 65°C media. Average temperature of 52°C over 18 s, "intermediate temperature profile". Recordings were taken at 0.1 ms intervals.

Effects of hyperthermia on airway smooth muscle and epithelial cells

To determine the effect of hyperthermia on individual structural cell types *in vitro*, growth medium was heated to various temperatures equating to bronchial thermoplasty, as discerned from the thermal profiling studies, and transferred to chamber slides containing cultured ASM cells or HBE cells. By testing different media temperatures and observing the thermal profiles, we determined that media heated to 85°C and added to a cell chamber incubated at 37°C best replicated the thermal profile seen at the bronchial thermoplasty contact points, referred to as the "maximal temperature profile". The cell chamber temperature spiked to 72°C, dropped rapidly to 65°C and declined more slowly with an average temperature of 61°C, compared to an average temperature of 64°C in the pig airway (figure 2c). Medium heated to 65°C and added to the cell chamber best replicated the thermal profile seen in between the bronchial thermoplasty contact points, referred to as the "intermediate temperature profile". The cell chamber temperature spiked to 60°C and to 52°C (compared to an average of 55°C in the pig airway; figure 2d). Medium heated to 37°C was added to the cell chambers as control.

Exposure of ASM cells to the intermediate temperature profile had no effect on cell morphology at 24 h post-exposure when compared to control (figure 3a–d). However, exposure to the maximal temperature profile produced marked cytoplasmic disruption, condensation of nuclei and loss of cellular adhesion at 24 h post-exposure (figure 3e, f). In contrast, exposure to heated media had no effect on HBE cell morphology (data not shown).

There was a significant reduction in ASM cell viability in response to both the maximal and intermediate temperature conditions 0.5 h post-exposure; viability recovered after 3 and 24 h of cell culture in the intermediate temperature group, but remained submaximal at 3 and 24 h in the maximal temperature group (figure 4).

The effect of bronchial thermoplasty on airway remodelling

68 biopsy samples obtained from the nine study participants (baseline n=24, 6 weeks n=22, 12 months n=22) were adequate for analysis (figure 1). Each biopsy was analysed independently, and the results averaged for each patient at each time point to achieve a single data point. ASM expressed as a percentage of the submucosal area was significantly reduced at 6 weeks and 12 months (baseline 12.05±1.03%, 6 weeks 4.40±1.30%, 12 months 5.07±1.66%; figure 5a and 6a). Collagen deposition, defined as collagen present within the submucosa, beneath and excluding the sub-basement membrane [32, 35, 36], expressed as a percentage of total submucosal area, was significantly, but transiently reduced at 6 weeks following bronchial thermoplasty (baseline 2.47±0.33%, 6 weeks 1.16±0.21%, 12 months 1.63±0.30%; figure 5b and 7a). The dominant red birefringent collagen present at baseline was replaced by increased green birefringent collagen at 6 weeks post-bronchial thermoplasty, but reverted to the baseline pattern at 12 months post-bronchial thermoplasty (figure 5b and 7b, c). Average vascular area and vascular density, representing size and numbers of vessels, respectively, expressed as a percentage of submucosal area, were increased at 6 weeks following bronchial thermoplasty, but subsequently reverted to baseline levels at 12 months (vascular area: baseline 27 600±7672 μm^2, 6 weeks 66 664±15083 μm^2, 12 months 45 921±11819 μm^2; vascular density: baseline 3.54±0.48%, 6 weeks 5.85±0.61%, 12 months 3.75±0.77%; figure 7d, e). Bronchial thermoplasty significantly reduced nerve bundles identified in the submucosa at 6 weeks post-bronchial thermoplasty and 12 months (baseline 9.457±3.405, 6 weeks 3.585±1.821, 12 months 2.460±0.803; figure 5c and 6b). There was no significant difference in goblet cell metaplasia, sub-basement membrane thickness or area of submucosal glands (supplementary figure S1b and c) following bronchial thermoplasty at any time point.

The effect of bronchial thermoplasty on clinical measurements

Patient demographics and baseline measurements prior to bronchial thermoplasty are in table 1. Clinical follow-up did not reveal significant changes in Asthma Control Questionnaire (ACQ), Asthma Quality-of-Life Questionnaire (AQLQ), forced expiratory volume in 1 s (FEV_1) or forced vital capacity (FVC) following bronchial thermoplasty (figure 8). However, using the Chi-squared test of proportion to compare 12 months to baseline, six out of nine participants had an improvement in the ACQ score greater than the minimal clinically important difference of 0.5, while only one had a >0.5 deterioration (difference 55%, 95% CI 10.5–78.7; p=0.02). A similar trend was seen for AQLQ improvement in four out of nine *versus* deterioration in one out of nine, but this was not statistically significant. This resulted in a significant correlation between ASM and ACQ (using all data points, thereby accounting for change; r=0.408, p=0.048; no other significant correlations between clinical metrics and airway remodelling were demonstrated). No significant differences were noted for minimal clinically important difference changes in FEV_1 or FVC at 12 months (defined as a change of ⩾12% and 200 mL).

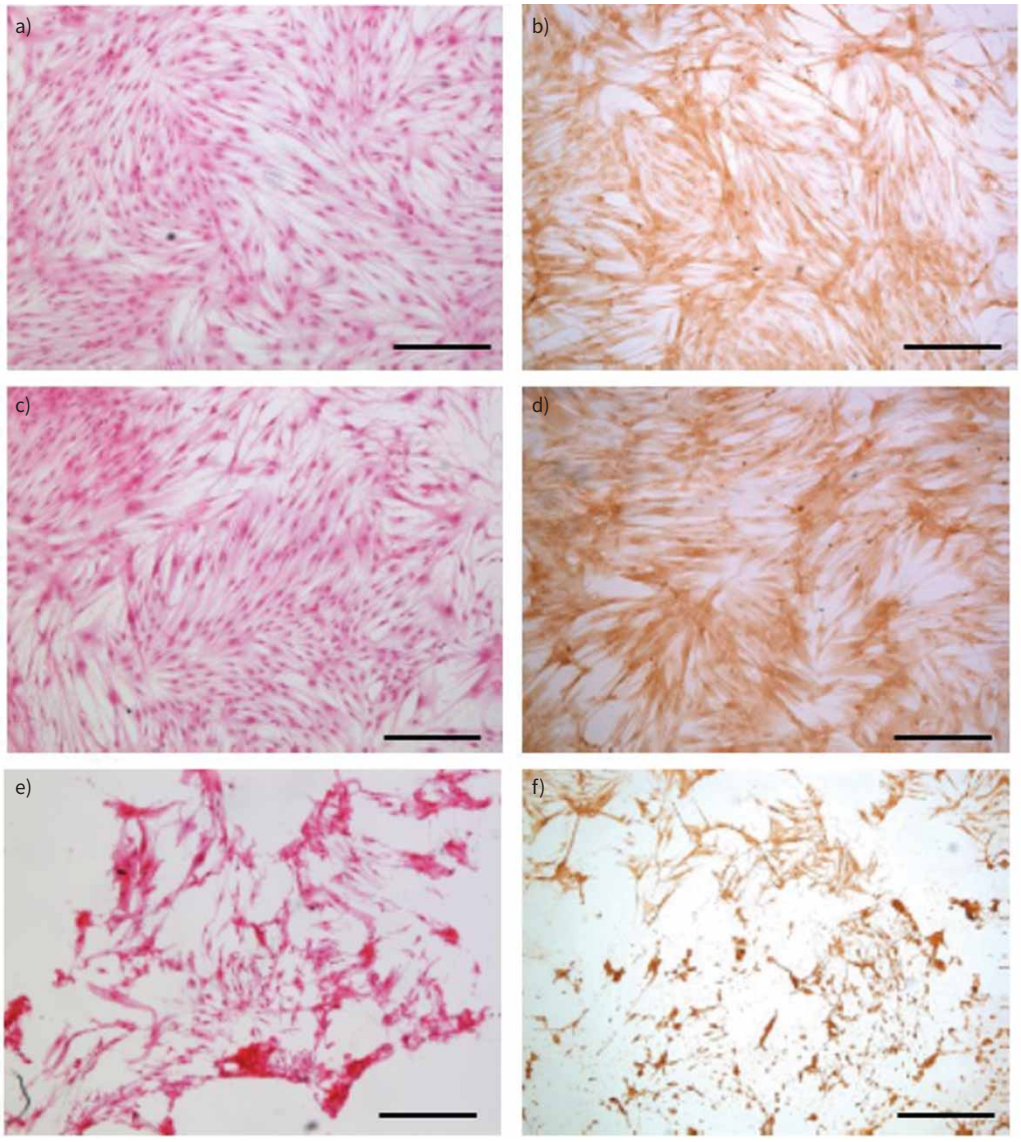

FIGURE 3 Effect of hyperthermia on airway smooth muscle cells. Airway smooth muscle (ASM) cell chambers at a temperature of 37°C were exposed to a, b) 37°C medium (control); c, d) 65°C medium ("intermediate temperature profile", average temperature 52°C); or e, f) 85°C medium ("maximal temperature profile", average temperature 64°C) for 10 s then cultured for an additional 24 h. a–d) The ASM cells in the control and intermediate temperature profile groups display normal morphology and staining characteristics. e, f) However, cells in the maximal temperature profile demonstrate disrupted cytoplasm, condensed nuclei, poor integrity of cell–cell contact and loss of α-smooth muscle acting (SMA) staining. n=4. a, c, e) Haematoxylin and eosin; b, d, f) α-SMA. Scale bars=20 μm.

Discussion

In order to determine the temperature profiles appropriate to use in our cell culture experiments, we examined the thermal profile of bronchial thermoplasty in a porcine model using a thermal camera. The porcine tracheobronchial system shares morphological characteristics with that of the human, including similar tracheal length, bronchiolar segmentation, and bronchiolar diameter. Moreover, both porcine and human bronchioles have a highly differentiated pseudostratified epithelial lining and submucosa with submucosal glands and airway smooth muscle [25, 37, 38] making the pig lung the favoured animal model to train bronchoscopists [39–41] and therefore the best choice to model bronchial thermoplasty in the human. We found the maximal temperature of the airway wall was ~83°C at the bronchial thermoplasty contact points; significantly higher than the intervening wall which reached a maximum of only 60°C.

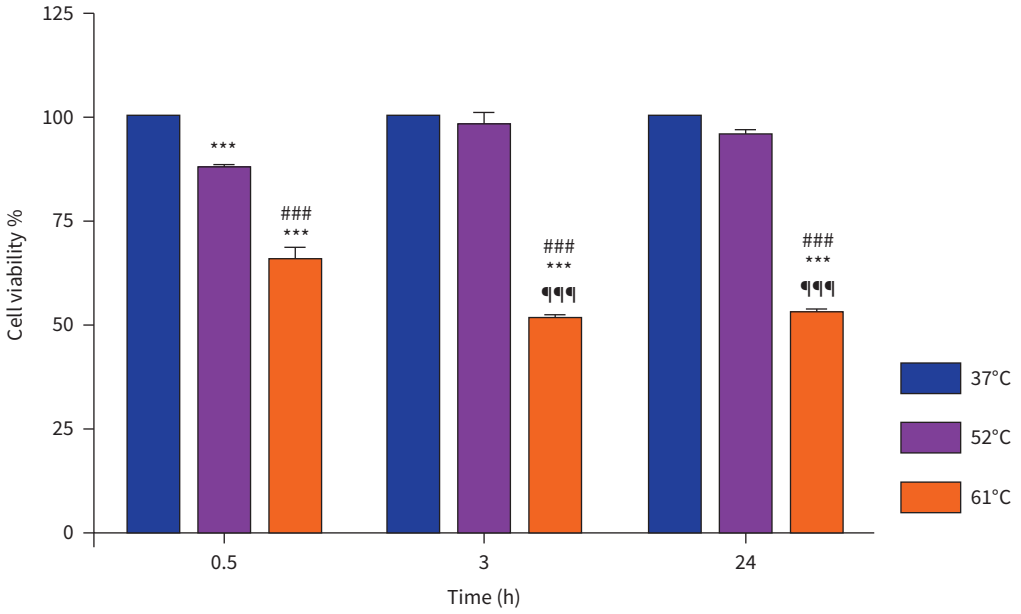

FIGURE 4 Airway smooth muscle (ASM) cell viability. A significant decrease in cell viability was seen in ASM cells heated to an average temperature of 52°C and 64°C compared to control at 0.5 h post-treatment, but those heated to 52°C had recovered viability to normal levels by 3 h and 24 h post-treatment. Cells heated to 64°C had further loss of viability compared to that at 0.5 h at 3 h and 24 h post-treatment. *Post hoc* ***: p<0.001 compared to control; ###: p<0.001 compared to cells heated to average 52°C at the same time points; ¶¶¶: p<0.001 compared to corresponding value at 0.5 h.

It is possible that some variations in morphology in the pig compared to the human bronchioles may alter heat distribution. Additionally, it is possible that the spike in temperature in a live animal (or human) is likely to be more transient as blood flow in an intact lung would redistribute the applied heat. However, using excised airways was a necessary adaptation to allow imaging. The average temperatures at the contact points and in the intervening wall were 64°C and 55°C, respectively, and are consistent with the findings of Chernyavsky *et al.* [42], who found that the temperatures were ⩾65°C and ⩽55°C at and between the contact points, respectively. The heterogeneous heating by bronchial thermoplasty may explain, at least in part, why a circumferential reduction in ASM of only ∼50% has been demonstrated [43] and the variability in other histological measures that have been reported to date.

High-frequency electrical energy, without heat generation (rheoplasty), has been shown to affect bronchial epithelium, with a reduction in goblet cell metaplasia [44]. In the case of bronchial thermoplasty, the radiofrequency energy translates to thermal energy in tissues. Hyperthermia affects cell membrane stability and impairs transmembrane transport protein and extracellular surface receptor function as well as proteins involved in DNA replication and stability [23, 24]. Therefore, cells exposed to heat are susceptible to apoptotic and/or necrotic signalling [17]. Previously, ASM cells have been shown to be susceptible to heat with a loss of myofilaments being observed at >48°C [45] and a loss of ASM contractility at >55°C [46]. Cell cultures were exposed to maximal and intermediate temperature profiles, to represent the situation at and between bronchial thermoplasty contact points, respectively. Similar to our study, in which we found that 61°C for 10 s disrupted ASM cells, Chernyavsky *et al.* [42] found irreversible loss of ASM cell viability with 59°C for 10 s. In addition, we found that HBE cells were relatively resistant to thermal injury when analysed 24 h after heat application, consistent with Chernyavsky *et al.* [42].

A characteristic of airway remodelling, and a major distinguishing feature of severe asthma, is increased ASM [3–5]; therefore, any reduction of ASM is potentially of clinical benefit. Previous studies have demonstrated a reduction of ASM in severe asthmatic patients after bronchial thermoplasty in the short term [10, 11, 13–15], which are now substantiated by the Bronchial Thermoplasty Induced Airway Smooth Muscle Reduction and Clinical Response in Severe Asthma (TASMA) trial, which, to date, is the only bronchial thermoplasty trial demonstrating ASM reduction 6 months post-bronchial thermoplasty in comparison to standard care [15]. We found a similar reduction in ASM in the short-term, but extend these

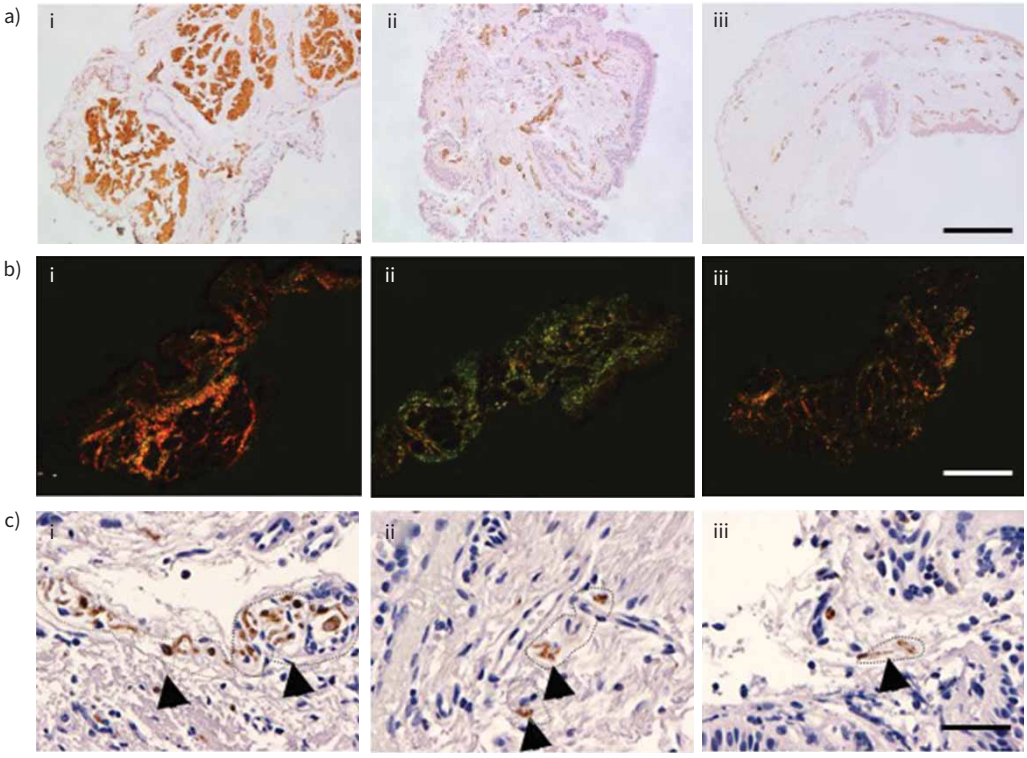

FIGURE 5 Representative biopsies at baseline and follow-up. a) Bronchial biopsies demonstrating airway smooth muscle at i) baseline, ii) 6 weeks and iii) 12 months following bronchial thermoplasty. Brown stain positive for α-smooth muscle actin. Scale bar=200 μm. b) Sections stained with picrosirius red and visualised using polarised light to demonstrate submucosal collagen deposition. Note that collagen shows a majority of red birefringence at i) baseline and iii) 12 months, while the majority of the collagen displays green birefringence at ii) 6 weeks. Scale bar=200 μm. c) Sections immunostained with anti-S100 (brown stain) demonstrate the reduction of nerve fibres (arrowhead, outlined) at i) baseline, ii) 6 weeks and iii) 12 months following bronchial thermoplasty. Scale bar=20 μm.

findings and confirm the report by others [10, 15, 16], by demonstrating a sustained and significant reduction in ASM at >12 months post-bronchial thermoplasty.

Our findings support the safety of bronchial thermoplasty with regard to the airway epithelium [19, 21, 47]. Although our *in vitro* studies with HBE cells suggest that bronchial thermoplasty does not adversely affect the bronchial epithelium, we cannot rule out an early, short-term effect. Using optical coherence tomography (OCT) to examine the airways immediately after bronchial thermoplasty, Goorsenberg *et al.* [43] reported that bronchial and peribronchial oedema and sloughing of the epithelium were seen in the majority of the treated airways. Oedema was also seen in distal nontreated airways. On repeating OCT 6 weeks later, the mucosa was essentially re-epithelialised, and the oedema had mostly receded. This is consistent with the demonstration of increased epithelial integrity in biopsies attained 3 months post-bronchial thermoplasty [42]. Interestingly, culture of epithelial cells harvested from patients have demonstrated increased proliferation post-bronchial thermoplasty, where the authors suggested that effects on remodelling post-bronchial thermoplasty relate to reduced epithelial stimulation of fibroblasts [48]. Although we did not identify any alterations in airway epithelial cell morphology nor increase in goblet cell metaplasia (supplementary figure S1), it is plausible that bronchial thermoplasty affects the cross-talk between epithelial cells and underlying fibroblasts and smooth muscle. We did not find an effect on sub-basement membrane thickness, in contrast to other studies [10, 11].

We were concerned that thermal injury to the airway wall could give rise to scar formation and therefore assessed the effect of bronchial thermoplasty on submucosal collagen. We found a significant reduction in collagen deposition in the submucosa at 6 weeks post-bronchial thermoplasty, which then returned to baseline levels by 12 months post-bronchial thermoplasty (figure 7a). We are the first investigators to

assess submucosal collagen after bronchial thermoplasty. We speculate that the apparent reduction in submucosal collagen can be explained by the presence of a wound repair response with oedema fluid pushing apart the collagen fibres. Interestingly, there was also a transient but significant increase in vascular density at 6 weeks which may represent new, "leaky" vessels as seen in wound repair (figure 7d) [49]. Collagen staining with PSR exhibits different colours under polarised light, including red, green and yellow. There is debate whether these different colours represent different types of collagen, as proposed by JUNQUEIRA *et al.* [50]. who claimed that type I collagen shows red birefringence whereas type III collagen appears green [33, 50, 51]. However, other studies dispute this claim, suggesting that these variations in birefringence are related to the thickness of the collagen fibrils only [51]. Whether or not the colours represent specific types of collagen, it is apparent that there was a significant change at 6 weeks compared to baseline, coinciding with the other changes which suggest a wound repair response [33, 34]; this appeared to have resolved at 12 months post-bronchial thermoplasty (figure 7b, c). A previous study showed a reduction in vessel number at 6 weeks post-bronchial thermoplasty which did not reach significance, while another showed no difference at 3 months post-bronchial thermoplasty [10, 13]. The vessels at the 12-month time point showed no changes compared to baseline, in contrast to the fact that a significant increase in the number of vessels and/or percentage of vascular area in the airways of subjects with asthma has been observed in comparison to healthy controls [52] and correlates with bronchial hyperresponsiveness [53].

The nerves in the airway submucosa are part of the parasympathetic nervous system and are critical to the regulation of smooth muscle tone and contribute to the pathophysiology of bronchospasm [54]; they have also been implicated in the regulation of epithelial mucus production [55]. We found significantly decreased numbers of nerve bundles at 6 weeks and 12 months post-bronchial thermoplasty in comparison to baseline (figure 6b), a finding that is in keeping with other authors who found similar reduction in nerves at 12 months post-bronchial thermoplasty [10, 22].

The morphological changes which occur as a result of bronchial thermoplasty are key structural features responsible for AHR [5, 7, 8]. The attenuation of airway remodelling is consistent with preclinical data which demonstrate reduction of airway hyperresponsiveness in dogs [56]. The airway dysfunction that results from airway hyperresponsiveness is important in the pathophysiology of asthma morbidity and occasional asthma mortality [57, 58], and previous clinical reports have demonstrated reduced hospitalisations and exacerbations following bronchial thermoplasty [19–21]. Although we did not specifically assess airway hyperresponsiveness in this study, we found no change in pre-bronchodilator FEV_1 and FVC, consistent with previous results [19–21] that showed no significant alteration of lung function following bronchial thermoplasty.

This study was not powered to identify clinical improvement and we did not detect either a statistically significant or clinically meaningful change in the AQLQ score, but did demonstrate a clinically relevant

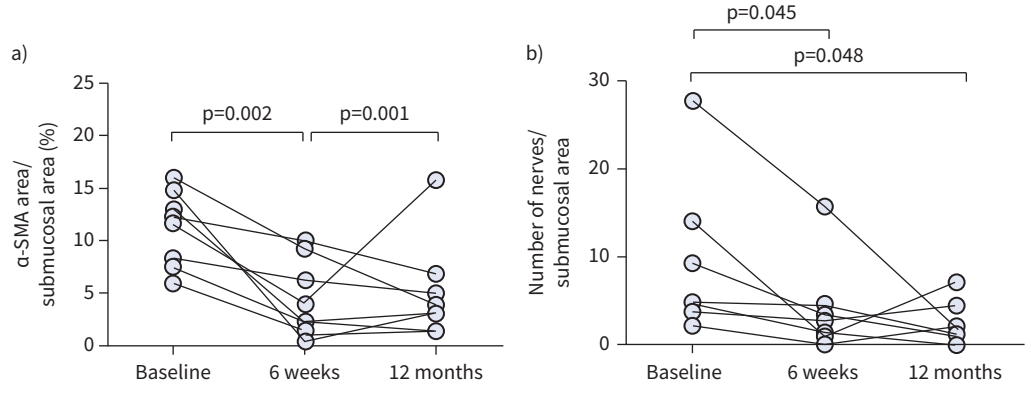

FIGURE 6 Histological evaluation of smooth muscle and neural innervation following bronchial thermoplasty. a) Smooth muscle area normalised to submucosal area was significantly reduced 6 weeks and 12 months after bronchial thermoplasty compared to baseline (ANOVA F=11.817, p<0.001). b) Nerve density normalised to submucosal area was significantly reduced at 6 weeks and 12 months post-bronchial thermoplasty compared to baseline (ANOVA F=3.959, p=0.048). *Post hoc* p-values demonstrating significant comparisons are indicated. SMA: smooth muscle actin.

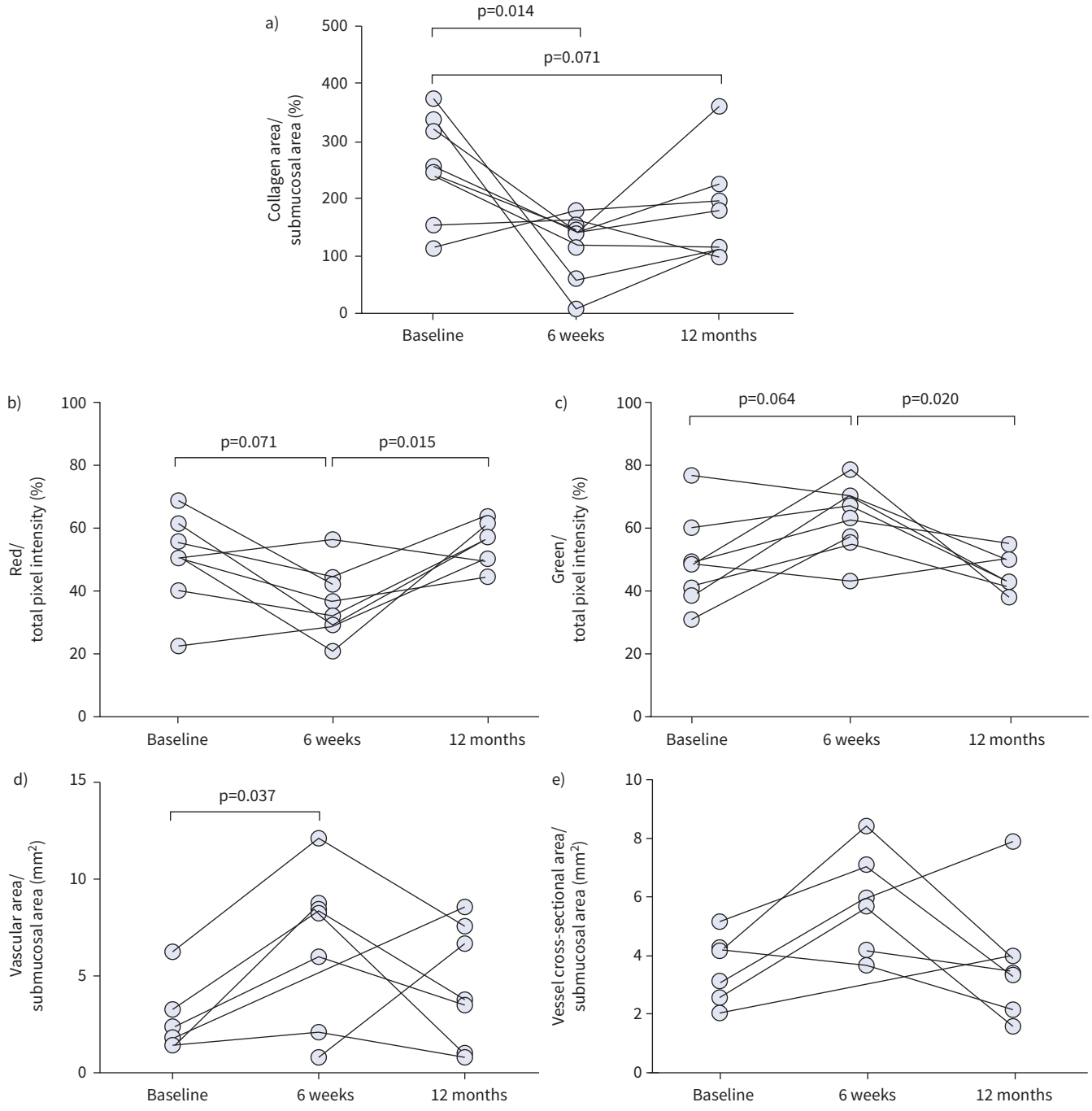

FIGURE 7 Histological evaluation of collagen and vascularity following bronchial thermoplasty. a) Collagen area normalised to submucosal area was significantly reduced at 6 weeks, but the difference was no longer significant at 12 months, although there was a trend to a reduction in collagen between baseline and 12 months (ANOVA F=5.536, p=0.017). b) Type I collagen (displaying predominantly red birefringence under polarised light) expressed as a percentage of total collagen was reduced at 6 weeks and recovered at 12 months following bronchial thermoplasty (ANOVA F=5.508, p=0.012). c) Type III collagen (displaying predominantly green birefringence under polarised light) was augmented at 6 weeks and subsided at 12 months (ANOVA F=5.269, p=0.015). d) Vascular area normalised to submucosal area was significantly increased at 6 weeks, but returned to baseline levels at 12 months (ANOVA F=4.175, p=0.048). e) Vascular density showed a non-significant trend towards an increase at 6 weeks and subsequent reduction at 12 months following bronchial thermoplasty (ANOVA F=3.636, p=0.065). *Post hoc* p-values demonstrating significant comparisons are indicated.

(>0.5-unit reduction) long-term improvement in ACQ, in accord with the Asthma Intervention Research (AIR), AIR2, Research in Severe Asthma and TASMA trials [15, 19–21, 47]. These data complement previous reports that the number of symptom-free days increased and severe exacerbations decreased

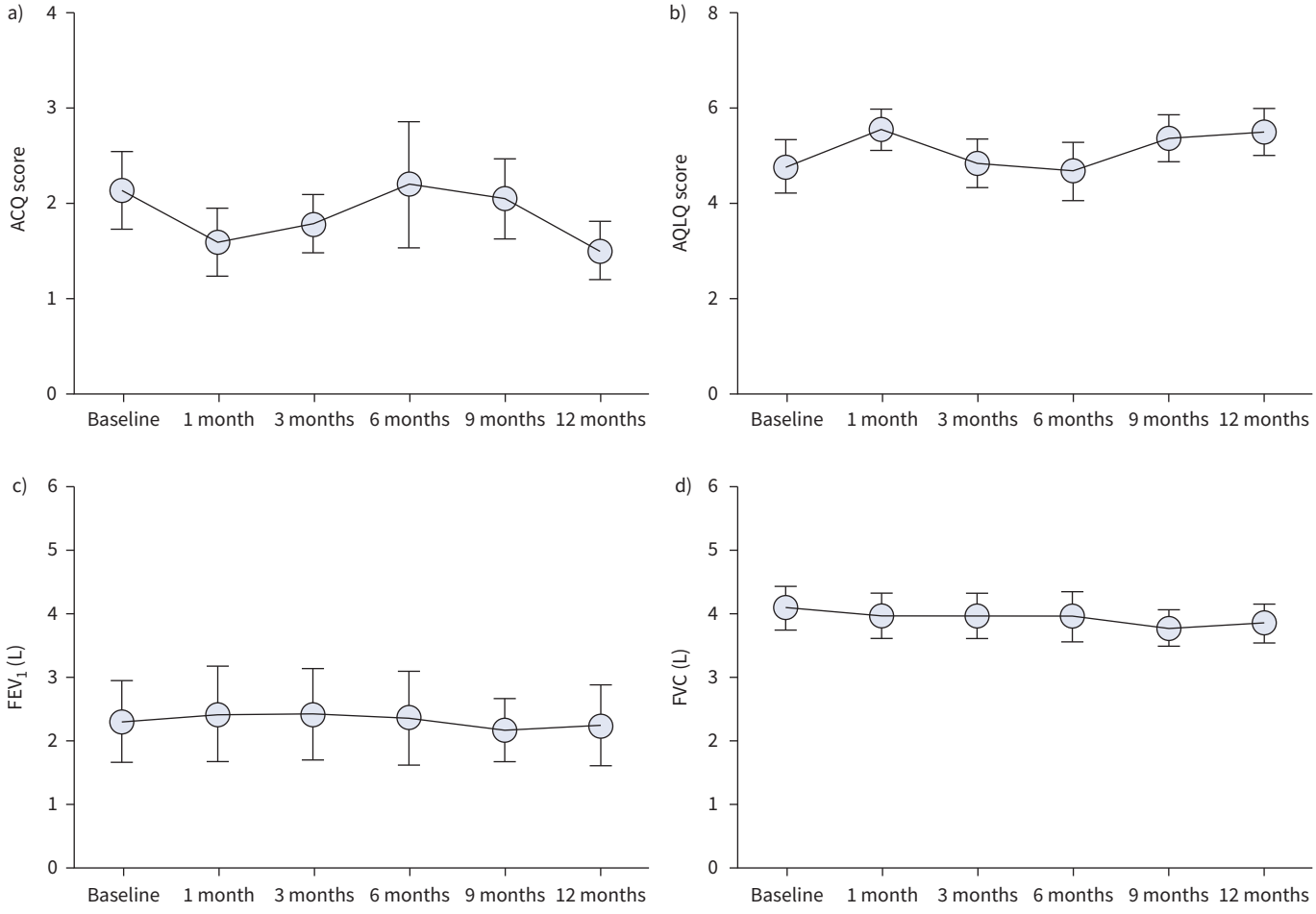

FIGURE 8 Clinical assessment at baseline and follow-up. a) Asthma control questionnaire (ACQ) was not statistically different between baseline or follow-up (n=9; 1 missing test at 3 months). However, compared to baseline, 12-month follow up demonstrated a clinically significant (>0.5) improvement with a statistical trend towards significance of improved asthma control. b) Asthma Quality-of-Life Questionnaire (AQLQ) was not statistically different between baseline or follow-up (n=9, n=1 missing test at 3 months). c) Forced expiratory volume in 1 s (FEV_1) was not improved at any time point prior to bronchial thermoplasty (n=9). d) Forced vital capacity (FVC) was not improved at any time point prior to bronchial thermoplasty (n=9).

following bronchial thermoplasty treatment [15, 19]. ASM reduction is significantly correlated with asthma control, demonstrating the clinical significance of the effect of bronchial thermoplasty on ASM [13]. However, in contrast, the change of ACQ attributed to bronchial thermoplasty in the TASMA trial was correlated with baseline plasma eosinophils, not ASM [15], suggesting that inflammation and signals to stimulate ASM contractility may be more indicative of exacerbations than ASM itself. Similarly, yet another study demonstrated clinical improvement in both ACQ and AQLQ 12 months following bronchial thermoplasty along with a reduction of lung neural innervation; however, a clear relationship between clinical improvement and reduced lung nerves was not assessed [22]. Although we did not find statistically significant differences in our clinical findings, we did demonstrate that ASM and ACQ were significantly correlated (r=0.408, p=0.048) in line with Ichikawa *et al.* [13], as well as clinically meaningful improvements in six out of nine patients with ACQ.

A limitation to our study is the lack of a control/sham group. Incorporation of a sham group was not feasible, as the prospect of withholding treatment would reduce enthusiasm for enrolment and provide additional ethical concerns. However, the main objective of our study was to examine airway remodelling and not clinical improvement, and follow-up assessment of airway hyperresponsiveness was not part of this study.

In conclusion, this is the first study to systematically examine the temperature profile of bronchial thermoplasty on the airway wall *in situ* and observe the effect of this heat profile on smooth muscle and

epithelial cells. We found that bronchial thermoplasty heats the airway wall unevenly, depending on the proximity to the bronchial thermoplasty catheter wires, and that peak temperatures were higher than expected. Heat had a significant deleterious effect on ASM morphology and viability, while bronchial epithelial cells were relatively resistant. We also observed a significant reduction in ASM and numbers of submucosal nerve bundles, which were sustained up to 12 months following bronchial thermoplasty with no observed changes in goblet cell metaplasia, the thickness of the sub-basement membrane or the submucosal glands. We did find a transient decrease in submucosal collagen, which had different birefringent properties, and an increase in vessel density which we speculate may represent a wound repair response at 6 weeks post-bronchial thermoplasty. However, we found no evidence of scarring at the 1 year post-bronchial thermoplasty time point.

Presently, bronchial thermoplasty is the only therapy which has been shown to reverse certain indices of airway remodelling, namely smooth muscle volume and numbers of nerve bundles, in severe asthma. Our study confirmed this and identified that, although there may be a wound repair response 6 weeks after bronchial thermoplasty, this is transient, with no evidence of scarring at 12 months post-bronchial thermoplasty, a reassuring finding with regard to safety with this treatment.

All de-identified data are available from the corresponding authors upon reasonable request.

Author contributions: N. Jendzjowsky conducted morphometry and analysis, prepared figures and the manuscript; A. Laing conceptualised the study, conducted morphometry, experiments, analysis and, prepared figures; M. Malig conducted morphometry and analysis; J. Matyas assisted with morphometry; E. de Heuvel prepared samples, helped with experiments and contributed to manuscript preparation; C. Dumonceaux recruited participants and collected clinical data; E. Dumoulin conducted bronchial thermoplasty and collected samples; A. Tremblay conceptualised the study, conducted bronchial thermoplasty, collected samples, coordinated patient selection and contributed to manuscript preparation; R. Leigh conceptualised the study, coordinated patient recruitment, and contributed to manuscript preparation; A. Chee conceptualised the study, obtained funding, conducted bronchial thermoplasty and collected samples; M.M. Kelly conceptualised the study, coordinated histology, morphometry, experiments and immunohistochemistry and contributed to manuscript preparation. All authors approved the final version of the manuscript.

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