

Airway smooth muscle proliferation and inflammation in asthma

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ABSTRACT

32 In asthma, it is unclear if the airway smooth muscle cells proliferate more or are increased at the
33 onset of asthma and remain stable. This study aimed to compare smooth muscle cell proliferation in
34 individuals with and without asthma and correlate proliferation rates with cell size and number and
35 with granulocytic airway inflammation.

36 Post-mortem airway sections were labelled with proliferating cell nuclear antigen (PCNA) and
37 percent positive muscle cells calculated. On the same sections, smooth muscle cell size and number,
38 and the number of eosinophils and neutrophils were estimated and compared in cases of nonfatal
39 (n=15) and fatal (n=15) asthma, and control subjects (n=15).

40 The %PCNA⁺ muscle cells was not significantly different in fatal ($29.4 \pm 7.7\%$, mean $\pm$ SD),
41 nonfatal asthma ($28.6 \pm 8.3\%$) or control subjects ($24.6 \pm 6.7\%$) and not related to mean muscle cell
42 size ($r=0.09$), number ($r=0.36$), thickness of the muscle layer ($r=0.05$) or eosinophil numbers
43 ($r=0.04$) in the asthma cases.

44 These data support the hypothesis that in asthma the increased thickness of the smooth muscle layer
45 may be present before or at the onset of asthma and independent of concurrent granulocytic
46 inflammation or exacerbation.

47

48 **New and noteworthy:**

49 There is debate regarding the origins of the increased airway smooth muscle in asthma. It may be
50 independent of inflammation or arise as a proliferative response to inflammation. The present study
51 found no increase in the proportion of proliferating smooth muscle cells in asthma and no relation
52 of proliferation to numbers of airway smooth muscle cells or inflammation. These results support a
53 stable increase in smooth muscle in asthma that is independent of airway inflammation.

54 **Key words:** Hyperplasia; hypertrophy; morphometry; proliferating cell nuclear antigen.

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58 INTRODUCTION

59 Patients with asthma have variable symptoms including breathlessness, chest tightness and wheeze,
60 which result from excessive airway narrowing. Asthma is characterized pathologically by airway
61 inflammation and remodeling of the airway wall with less involvement of the lung parenchyma.
62 The most striking and consistent aspect of airway wall remodeling in asthma is the increased
63 thickness of the airway smooth muscle (ASM) layer (4). In cases of mild, moderate and severe
64 asthma, the thickness of the ASM layer is increased and the increased thickness is related to asthma
65 severity but not to the duration of asthma or to age (13). In addition, the increased thickness of the
66 ASM layer is due to both hyperplasia and hypertrophy, without a change in the proportion of
67 extracellular matrix, all of which are related to asthma severity but not to duration of asthma (14).

68
69 The origins of increased ASM in asthma remain unclear. Hypotheses include: early-life or prenatal
70 effects on ASM development leading to increased volume of ASM which persists through life and
71 interacts with inflammation; proliferation and hyperplasia driven by an altered phenotype of ASM
72 or by inflammation / allergy / sensitisers or a primary defect in the respiratory epithelium leading to
73 epithelial-mesenchymal transformation. These hypotheses might be summarised as being either
74 “parallel” - those that suggest an independent role of a stable increase in ASM (the degree of which
75 determines the severity of asthma) interacting with inflammation to cause excessive airway
76 narrowing or “serial” - those that suggest a linear progression of inflammation constantly driving
77 airway remodeling. Proliferation of ASM would be expected to be stable in the parallel model and
78 increased in the serial model. Since either has profound importance in addressing the origins (and
79 potential treatments) of increased ASM in asthma, knowledge about the proliferation of ASM in
80 patients with asthma is vital.

81
82 Although ASM cells proliferate *in vitro* and more so from patients with asthma (17), studies using
83 bronchial biopsies of the central airways have reported conflicting results, showing no increase in

proliferation in some studies (2,34,11) but increased proliferation in others (8, 29) in asthma cases compared with non-asthmatic controls. Recently, the proliferative activity of ASM in culture, driven by matrix metalloproteinase-1 and mast cell protease, was shown to be increased during exacerbations of asthma (24) and a strong regional relationship between airway wall remodeling and airway inflammation has been noted (7). Therefore it is possible that intermittent increased proliferation may be associated with exacerbations and inflammation. To address the relation of proliferation to asthma severity, exacerbations (fatal attack), airway inflammation and the number of ASM cells within the airway wall, we compared ASM proliferation within the airway wall in fatal and nonfatal cases of asthma and in control subjects without asthma. Unlike previous studies, where proliferation was assessed in culture or after bronchial biopsy, we assessed proliferation in the intact airway wall using serial cross sections, thereby preserving the ASM in situ.

METHODS

Subjects

Subjects included in this study were from a multi-centre collection of tissue - the Airway Disease Biobank - housed within our laboratory and described in a previous publication (14). Information regarding history of asthma and where available, age of onset of asthma, frequency of asthma symptoms, use of asthma medications, smoking history, lost time from work or school due to asthma, hospitalisation due to asthma and measurements of lung function were obtained from next-of-kin, hospital files, coroners' files and the subjects' usual medical practitioners. Subjects were categorised as: Controls - no history of asthma, wheeze or other lung disease; Nonfatal Asthma - died suddenly of non-respiratory causes but with a confirmed history of asthma, and; Fatal Asthma - died from asthma with a confirmed history. Approval for this study was obtained from all the Institutional Ethics Committees of the participating centres and from the Sir Charles Gairdner Hospital Human Research Ethics Committee.

110 **Tissue preparation**

111 Post-mortem tissue samples were fixed in inflation or by immersion using formaldehyde or
112 glutaraldehyde and embedded in paraffin. Airways were sampled using a systematic sampling
113 design or for diagnostic purposes at the discretion of the pathologist. Sections were cut at 0.5, 4 and
114 30µm thickness from each block and stained using the Masson's trichrome technique, haematoxylin
115 and eosin, and haematoxylin respectively.

116

117 **Immuno-histochemistry**

118 An additional section was cut at 4µm and labelled with proliferating cell nuclear antigen (PCNA).
119 Deparaffinization and antigen retrieval was undertaken by placing sections in a pre-treatment unit
120 (PT Module, Thermo Scientific (A80410200) Runcorn, United Kingdom) at 100 degrees for 36 min
121 in ethylene-diamine-tetraacetic acid (EDTA, pH8). Sections were then washed in Tris-buffered
122 saline buffer (TBS, pH7.5) and treated with 3% hydrogen peroxide for 10 minutes to remove
123 endogenous peroxidase activity. Sections were then washed in TBS buffer and PCNA antibody
124 (Dako M0879) applied at a dilution of 1:100 and incubated for 1hr at 36 degrees. EnVision
125 detection system was used followed by DAB substrate chromogen (Dako K4007). Sections were
126 counter-stained with haematoxylin, dehydrated with xylene and a coverslip applied. TBS buffer was
127 used for the negative control.

128

129 **Airway measurements**

130 On 4µm sections stained with haematoxylin and eosin, the inner airway wall area (W_{Ai} - between
131 the basement membrane and the outer border of the ASM layer) and the perimeter of the basement
132 membrane (P_{bm}) were measured. The P_{bm} was used to define airway size as large (P_{bm} >6mm) or
133 small (P_{bm} <6mm) (15). On the same slide, eosinophil and neutrophil numbers were counted
134 within the inner airway wall (between the basement membrane and the inner border of the ASM
135 layer), around the entire airway section, based on staining and morphology criteria. On 30µm

136 sections, the numerical density (N_V) of ASM cell nuclei was estimated using the optical disector
137 (18) and the ASM area (ASM_{area}) was measured. PCNA⁺ASM cells were counted by their nuclei
138 (ASM are cells mononuclear (6)), as their cell borders are indistinct and PCNA is a nuclear stain.
139 Airway dimensions and ASM cell parameters were estimated using stereological software
140 (newCAST version 4.2.1, Visiopharm A/S, Horsholm, Denmark) on airways cut in cross-section, as
141 previously described (14).

142

143 **Calculations**

144 The eosinophil and neutrophil area densities within the inner airway wall were estimated
145 (cells/WAi). The total number of ASM cells per mm length of airway (N_L) was calculated, $N_L =$
146 $N_V(\text{cells/mm}^3) \times ASM_{area}(\text{mm}^2) \times 1\text{mm length}$. Mean ASM cell volume (V_C) was calculated as the
147 inverse of the N_V , correcting for the volume fraction of matrix and other structures within the ASM
148 layer, as previously described (14). The number of PCNA⁺ASM cells within the ASM layer was
149 expressed as a percentage of the total number of ASM cells and also as an area density
150 (PCNA⁺ASM cells/ ASM_{area}).

151

152 **Data analysis**

153 Analyses were undertaken using SigmaPlot (Version 13.0, Systat Software Inc. GmbH, Germany).
154 Subject means were calculated for airways grouped by size since the thickness of the ASM layer is
155 influenced by airway size (4). All variables were analysed using analysis of variance (ANOVA) and
156 post hoc tests as appropriate. The relationships between PCNA⁺cells and ASM cell number and
157 size, and airway inflammation were tested using Spearman's rank correlation in control subjects,
158 nonfatal and fatal asthma cases separately and in all asthma cases combined. Results are shown
159 without correction for multiple comparisons.

160

161 **RESULTS**

162 **Subject characteristics**

163 The subject characteristics are shown in Table 1. There were no significant differences seen in
164 gender, weight, body mass index, ever having smoked cigarettes, corticosteroid use or asthma
165 severity between groups (Table 1). The cases of fatal asthma were taller than the nonfatal asthma
166 cases ($p \leq 0.05$) and were older compared with the ($p \leq 0.05$) control subjects.

167

168 **Airway smooth muscle layer and airway inflammation**

169 No significant differences in basement membrane perimeters (airway size) were observed between
170 case groups in either airway size group (Table 2). An average of 2 airways (range 1-5) was
171 measured per case. The thickness of the ASM layer was significantly increased in the large airways
172 of the fatal asthma cases compared with the nonfatal asthma cases and with control subjects and in
173 the small airways of the fatal asthma cases compared with control subjects ($p \leq 0.05$). Similar,
174 nonsignificant trends were observed in the large and small airways of the cases of nonfatal asthma.
175 The number of ASM cells per airway length (N_L) was significantly increased in the large airways
176 only of the fatal asthma cases compared with control subjects ($p < 0.05$). No significant differences
177 were seen in the mean airway smooth muscle cell volume (V_C) between the study groups. The area
178 density of eosinophils was increased in large and small airways in fatal asthma compared with the
179 nonfatal asthma cases and controls subjects. The neutrophil area density was increased in large
180 airways only in fatal asthma compared with the nonfatal asthma cases.

181

182 **Airway smooth muscle cell proliferation**

183 PCNA positivity was observed frequently within the layer of ASM and within the airway
184 epithelium (Figure 1) in control subjects and asthma cases. There was no significant difference in
185 the number of PCNA⁺ ASM cells within the ASM layer, expressed as the area density or percentage
186 of total ASM cells (Figures 2, 3, Table 2). In control subjects, there was a positive correlation

187 between the percent PCNA⁺ ASM cells and the thickness of the ASM layer in the small airways
188 ($r=0.68$, $p=0.03$) but not for other ASM parameters (Table 3). In the nonfatal asthma cases, there
189 was a positive correlation between the percent PCNA⁺ ASM cells and mean ASM cell volume (V_C)
190 in small airways ($r=0.64$, $p=0.03$) but not for other ASM parameters. No significant correlations
191 were observed between percent PCNA⁺ ASM cells and any of the ASM parameters in the cases of
192 fatal asthma. No correlations were observed between the percent PCNA⁺ ASM cells and any of the
193 ASM layer parameters when all the asthma cases were combined, or when the control and asthma
194 groups were combined (Table 3).

195

196 In control subjects, the percent PCNA⁺ ASM cells was significantly and positively related to the
197 area density of eosinophils ($r=0.65$, $p=0.04$) and the area density of neutrophils ($r=0.75$, $p=0.01$) in
198 the large airways (Table 3). The percent PCNA⁺ ASM cells was not related to inflammatory cells in
199 the asthma cases.

200

201 **DISCUSSION**

202 This study shows that the proportion of proliferating ASM cells is similar in cases of asthma and
203 control subjects and, in fatal cases of asthma, is not related to the thickness of the layer of ASM or
204 the size and number of ASM cells within the airway wall. In nonfatal cases of asthma there was a
205 relationship between ASM cell proliferation and the estimated mean ASM cell volume. No
206 relationship was found between proliferation of ASM cells and the number of eosinophils or
207 neutrophils within the airway wall in cases of asthma. In non-asthmatic control subjects, a
208 relationship between proliferation and thickness of the ASM layer and eosinophilic inflammation
209 was observed. This is the first published study using sections of the whole airway wall to relate
210 proliferation to inflammation in situ, in large and small airways. These findings suggest that in
211 asthma, proliferation of ASM is not increased or related to granulocytic inflammation and supports

212 the hypothesis that the increased numbers of ASM cells seen in asthma is independent of the
213 increased inflammation seen in asthma, and is stable and not progressive.

214
215 A number of published studies have compared ASM proliferation in asthma with that in non-
216 asthmatic subjects (Table 4). *In vitro*, using a variety of methods of detection, ASM cells from cases
217 of asthma have been shown to proliferate more rapidly than those from control subjects (17, 33).
218 Remarkably, this occurred not only in fresh samples but also after several passages. However
219 another study found no increase in proliferation of ASM cells *in vitro* (19). Given the proliferative
220 conditions of the *in vitro* studies, it is difficult to relate the proliferation rates to those that might be
221 seen *in situ* (39). Studies using biopsies from the large airways of cases of asthma have previously
222 suggested no increase in proliferation in cases of asthma, compared with control subjects (2, 34).
223 Using either Ki67 (2) or cyclin D (34) as markers of proliferation these studies both found no
224 positive staining within the layer of ASM in either control subjects or cases of asthma, despite
225 showing positive staining in the airway epithelium and the airway submucosa. Hassan et al. (8)
226 compared PCNA with Ki67 and showed that PCNA was more sensitive and that PCNA⁺ ASM cells
227 were increased in severe (but not mild-moderate) cases of asthma compared with control subjects.
228 Studies from the same group have shown increased proliferation in some cases of asthma (29) but
229 not others (11). Increased proliferation was not confirmed in a study (27) where biopsies were
230 obtained in preschool wheezers and compared in those who subsequently did or did not develop
231 asthma at school age. However the number of cases in that study was relatively small (n=3 with
232 asthma) and asthma was not severe.

233
234 Like the study of Hassan et al. (8), the present study showed a high level of proliferation in both
235 control subjects and cases of asthma. PCNA expression is increased early in cell division and
236 PCNA contributes not only to DNA replication, but also to DNA repair, cell cycle control,
237 chromatin remodeling (epigenetic modifications), chromatid cohesion and transcription, among

238 others (23). In a study of prostate cancer (36), expression of PCNA in prostatic cancer cells was
239 23%, compared with less than 5% in benign prostatic cells. That study used a cancer-specific
240 isoform of PCNA. Based on unpublished work discussed in the publication (36), discrepancies in
241 expression of PCNA in different tissues might be due to tissue handling prior to fixation,
242 particularly with regard to raised temperatures ($>4^{\circ}$). Using staining for non-cancer specific
243 isoforms of PCNA, high levels of expression of PCNA (53%) have been observed in pituitary
244 adenomas (31) and in normal human spermatocytes (46%) (32). Expression has been shown to be
245 much lower in cardiac myocytes from normal patients ($<3\%$) and increased, although not to the
246 range observed in the present study, in those with cardiomyopathy or myocarditis ($<5\%$) and those
247 with transplanted hearts ($<8\%$) (1). A PCNA index $>7\%$ was associated with a worse prognosis in
248 renal cell carcinoma (22). To explore this further we measured PCNA⁺ cells in the epithelium of
249 four of our cases (n=2 with fatal asthma, n=2 control) and expressed them as a percent of all
250 epithelial cells within the region of interest. We found percent PCNA⁺ epithelial cells were 58%
251 and 44% in the cases of asthma and 40% and 20% in the non-asthmatic controls. Thus, we achieve
252 high rates of PCNA⁺ cells (epithelium and ASM cells) using the technique described, as well as
253 showing a difference between asthmatics and non-asthmatics in proliferation of epithelial cells as
254 has been noted previously (2). Therefore there seems to be great variation in expression of PCNA
255 between tissue, related to disease, handling artefact and perhaps related to the isoform of PCNA that
256 is labelled. Like most studies, antibody to the PC10 clone of PCNA was used in the present study
257 and, as in the study of Hassan et al. (8) and O'Reilly et al. (27), we used the same processes in a
258 blinded (regarding cases and controls) protocol when staining tissues so that preparation artefact
259 seems unlikely to account for the observed variations in differences between groups (control and
260 asthma).

261
262 The clinical severity of asthma and the treatment required to control symptoms over long periods of
263 time are relatively stable (26). In infants destined to develop asthma, lung function is reduced at
264 birth (before the onset of allergy and asthma) (38) and persists into adult life (16) and is

265 proportional to the severity of asthma (30). Since remodeling of the ASM is related to the severity
266 but not duration of asthma (13), it seems likely that this remodeling is present before or close to the
267 onset of asthma. The development of sensitisation to aeroallergen is the biggest risk factor for the
268 development of childhood asthma (12) and allergy is associated with airway inflammation (5). The
269 findings of the present study support the hypothesis that a pre-existing abnormality of airway
270 structure (ASM remodeling) combines with independently-determined allergic inflammation to
271 result in excessive airway narrowing, as we have recently demonstrated in a mouse model of
272 remodeling and allergy-induced airway narrowing (35). Under these circumstances excess
273 proliferation of ASM would not be expected.

274

275 Airway inflammation is associated with tissue damage and the release of cytokines and growth
276 factors which may have a proliferative effect on ASM (28). Severe asthma is associated with
277 eosinophilic inflammation (37) and a strong regional relationship between ASM remodeling and
278 airway inflammation has recently been observed in asthma (7). A number of hypotheses have
279 related inflammation to remodeling. Inflammation that results from allergic sensitivity and/or
280 epithelial fragility and damage (20) could release factors such as granulocyte colony stimulating
281 factor and transforming growth factor- β which result in epithelial-mesenchymal transformation and
282 the differentiation and proliferation of sub-epithelial fibroblasts into myocytes which contribute to
283 ASM remodeling (10). Alternatively, the release of growth factors could stimulate in situ ASM
284 proliferation and remodeling. In this regard it has been observed that, *in vitro*, ASM cells from
285 patients with asthma show increased proliferative responses to mitogenic stimuli (17). Lastly, the
286 ASM cells may themselves promote inflammation. ASM cells from patients with asthma release
287 growth factors and matrix proteins that may stimulate cell proliferation via an autocrine loop (3).
288 Mast cells are plentiful in the layer of airway smooth muscle and their numbers and degree of
289 degranulation are increased in asthma. During exacerbations, release of mast cell granule products
290 may contribute to a pro-proliferative milieu (24).

291

292 Proliferation of smooth muscle may vary in response to inflammatory stimuli as shown by a study
293 of heaves in horses (an allergic airway response to mouldy hay characterized clinically as difficulty
294 breathing ('heaves') and increased airway resistance) which showed increased proliferation of ASM
295 cells within the airways during periods of illness (9). A follow-up study (21) from the same group
296 demonstrated that withdrawal of the stimulus resulted in marked improvement in airway
297 inflammation, but only partial improvement in the ASM remodeling, leaving the question of the
298 relation between inflammation and remodeling unresolved. Analogous results have been shown in
299 patients with asthma where treatment with inhaled corticosteroids resulted in decreased thickness of
300 the airway, assessed by CT scan, although not back to values seen in non-asthmatic control subjects
301 (25). There is evidence of pro-proliferative changes in the airway walls of patients with asthma
302 undergoing an exacerbation due to viral infection (24). Despite these interesting observations, the
303 absence of significantly increased proliferation in cases of fatal asthma (i.e. undergoing an
304 exacerbation) in the present study does not support increased proliferation during an exacerbation.

305

306 There are a number of potential limitations to the current study. Although the size of airway tissue
307 sampled is orders of magnitude greater than in biopsy studies, we recognize that true random
308 sampling of the airways was not undertaken. Depending on the original study design (13), sampling
309 of large airways varied from arbitrary samples for post-mortem diagnostic purposes to systematic
310 collection of all airways to the sub-segmental level. Samples from the lung periphery were chosen
311 by cutting parenchymal blocks "at random" or by sampling along a bronchial pathway to the lung
312 periphery. Subsequent selection of airway blocks to be used for this study was arbitrary, determined
313 to a large extent by availability of tissues which had been included in previous studies (13,14).
314 Therefore, although the possibility of bias persists, tissue selection was initially unrelated to studies
315 of proliferation or inflammation. Given that the tissues were obtained post-mortem, limited clinical
316 data (including medications) are available for most individuals in the study. It is possible that use

317 of medications may alter airway inflammation and proliferation rates. We did observe a significant
318 increase in the mean age of the cases. This likely reflects the different causes of death in these
319 groups (increased suicide and accidental death in the nonfatal and control groups). We have not
320 previously seen an effect of age on airway remodeling (13) and did not see a difference in
321 proliferation rates in the nonfatal and control groups which were similar in age. We did not see a
322 relation between inflammatory cells and proliferation in the cases of asthma. In our studies of ASM
323 in whole airway sections we have identified the layer of ASM using haematoxylin and eosin. This
324 does not specifically identify the contractile proteins in the smooth muscle cells and may therefore
325 over-estimate the area of the smooth muscle layer. Accordingly, we have previously compared
326 estimates of the area of ASM on contiguous sections stained with haematoxylin and eosin with
327 those using antibody to alpha-smooth muscle actin. We found no differences between the two
328 methods either in large or small airways from cases of asthma or non-asthmatic controls (18, Table
329 E1). Finally, it should be noted that we are observing rates of proliferation cross-sectionally and not
330 over time. Therefore we cannot exclude short-term but important changes occurring intermittently
331 in this relatively small sample of cases. There are no studies of the variation in *in situ* proliferation
332 rates over time.

333

334 In conclusion, the present study suggests that proliferation is not increased in either mild or severe
335 asthma or in cases of fatal asthma which are characterised by marked airway wall remodeling and
336 eosinophilic airway inflammation and undergoing an exacerbation. These findings support the
337 hypothesis that the increased smooth muscle in asthma is stable over time and independent of
338 inflammation.

339

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344

345

346

347 **Table 1**

348 **Subject characteristics**

	Control n=15	Nonfatal asthma n=15	Fatal asthma n=15
Gender, M/F	9/6	8/7	11/4
Age (years), Median (IQR)	17 (14 – 34)	19 (16 – 34)	43 † (18 – 57)
Height* (m), Mean ± SD	1.68 ± 0.08	1.59 ± 0.14	1.73 ± 0.12 ‡
Weight* (kg), mean ± SD	60 ± 11	59 ± 18	67 ± 13
Body Mass Index*, (kg/m²), mean ± SD	22 ± 5	23 ± 4	23 ± 4
Cigarette smoking ever*, percent	50	67	40

349

350 * = Incomplete data. † = p value <0.05 v control. ‡ = p value <0.05 v nonfatal asthma.

351 Abbreviations: F = female; IQR = interquartile range (25-75%); kg = kilograms; M = male; m =
352 meters; n = number; SD = standard deviation.

353

354

355

	Control n=15	Nonfatal asthma n=15	Fatal asthma n=15
Basement membrane perimeter (Pbm, mm)			
≥6mm	12.1 ± 6.7	13.9 ± 9.7	12.9 ± 5.2
Mean ± SD, (range)	(6.1 - 27.8)	(6.3 - 39.0)	(7.1 - 24.3)
<6mm	4.0 ± 1.7	3.5 ± 1.5	4.6 ± 2.6
Mean ± SD, (range)	(1.6 - 5.9)	(1.2 - 5.1)	(1.2 - 5.5)
ASM layer thickness (ASM _{area} /Pbm, mm)			
≥6mm	0.032 ± 0.016	0.043 ± 0.027	0.082 ± 0.039*,†
<6mm	0.014	0.018	0.028*
Median (IQR)	(0.008 - 0.020)	(0.011 - 0.030)	(0.017 - 0.046)
Airway smooth muscle cell number per airway length (N _L), mm, x10 ⁵			
≥6mm	0.84	1.19	1.83*
Median (IQR)	(0.55 – 1.24)	(0.78 – 2.13)	(1.12 – 4.17)
<6mm	0.25	0.18	0.44
Median (IQR)	(0.19 – 0.34)	(0.14 – 0.351)	(0.22 – 0.65)
Mean airway smooth muscle cell volume (V _C), μm ³ , x10 ³			
≥6mm	2.23 ± 0.61	2.79 ± 0.84	3.31 ± 1.22
Mean ± SD			
<6mm	2.45 ± 1.09	2.99 ± 0.83	2.35 ± 0.84
Mean ± SD			
PCNA ⁺ ASM cells, % of total ASM cells			
≥6mm	27.0 ± 5.4	29.8 ± 6.5	29.7 ± 7.0
Mean ± SD			
<6mm	29.9 ± 5.82	29.7 ± 5.4	23.5 ± 2.2
Mean ± SD			
PCNA ⁺ ASM cells, cells/mm ²			
≥6mm	2117	2859	2479

Median (IQR)	(1213 – 3671)	(1606 – 3086)	(1527 – 7533)
<6mm	1114	1706	1779
Median (IQR)	(759 – 2404)	(1560 – 2339)	(1202 – 3590)
Eosinophil density within the inner airway wall			
≥6mm	2.02 ± 2.81	3.23 ± 5.45	67.48 ± 85.05 *†
Mean ± SD			
<6mm	0.00	0.00	82.62 *†
Median (IQR)	(0.00 - 1.45)	(0.00 - 2.76)	(22.54 - 115.44)
Neutrophil area density within the inner airway wall			
≥6mm	3.87 ± 3.63	2.55 ± 2.58	8.15 ± 5.85 †
Mean ± SD			
<6mm	8.07 ± 7.38	6.87 ± 8.10	23.89 ± 19.00
Mean ± SD			

358 * = p <0.05 v control. † = p <0.05 v nonfatal asthma. Abbreviations: ASM = airway smooth
 359 muscle; IQR = interquartile range; kg = kilograms; m = meters; mm = millimeters; n = number;
 360 PCNA = proliferating cell nuclear antigen; SD = standard deviation.

361

362 **Table 3**

363 **Regression analysis of PCNA⁺ ASM cells and ASM layer components**

	Control n=15	Nonfatal asthma n=15	Fatal asthma n=15	All asthma n=30	All cases n=45
% PCNA ⁺ ASM cells v ASM _{area} /Pbm					
≥6mm	0.05 / 0.89	0.20 / 0.56	0.03 / 0.94	0.05 / 0.83	0.08 / 0.66
<6mm	0.68 / 0.03	0.13 / 0.71	0.35 / 0.49	0.24 / 0.36	0.06 / 0.75
% PCNA ⁺ ASM cells v ASM cell number per airway length (N _L)					
≥6mm	0.12 / 0.75	0.31 / 0.35	0.47 / 0.15	0.36 / 0.10	0.22 / 0.22
<6mm	0.34 / 0.33	0.26 / 0.43	0.33 / 0.52	0.30 / 0.24	0.11 / 0.59
% PCNA ⁺ ASM cells v ASM cell volume (V _C)					
≥6mm	0.16 / 0.66	0.06 / 0.84	0.21 / 0.51	0.09 / 0.66	0.16 / 0.35
<6mm	0.28 / 0.40	0.64 / 0.03	0.56 / 0.25	0.05 / 0.86	0.16 / 0.42
% PCNA ⁺ ASM cells v Eosinophils/(W _{Ai})					
≥6mm	0.65 / 0.04	0.48 / 0.09	0.17 / 0.60	0.04 / 0.85	0.13 / 0.46
<6mm	0.17 / 0.62	0.30 / 0.36	0.41 / 0.42	0.24 / 0.35	0.11 / 0.59
% PCNA ⁺ ASM cells v Neutrophils/(W _{Ai})					
≥6mm	0.75 / 0.01	0.46 / 0.11	0.10 / 0.76	0.17 / 0.40	0.01 / 0.94
<6mm	0.03 / 0.94	0.42 / 0.20	0.21 / 0.69	0.21 / 0.43	0.10 / 0.60
% PCNA ⁺ ASM cells v Age, (years)					
≥6mm	0.29 / 0.41	0.33 / 0.27	0.39 / 0.22	0.35 / 0.09	0.28 / 0.10
<6mm	0.04 / 0.91	0.32 / 0.34	0.24 / 0.65	0.07 / 0.78	0.03 / 0.87

364 r value / p value. Abbreviations: ASM = airway smooth muscle; PCNA = proliferating cell nuclear
365 antigen; W_{Ai} = inner airway wall.

366

368 **Summary of published studies of smooth muscle proliferation in asthma**

Reference	Sample	Asthma Severity	Markers of Proliferation	Proliferation in Asthma
Ijpma et al. (11)	PM, Biopsies	Mild, Moderate, Severe	PCNA, Cyclin D, Ki67	No
O'Reilly et al. (27)	Biopsies	Pre-school wheezers	PCNA	No
Hassan et al. (8)	Biopsies	Moderate - severe	PCNA	Yes, in severe cases of asthma
Ramos-Barbon et al. (29)	Biopsies	Moderate - severe	PCNA	Yes, in severe and moderate cases of asthma
Ward et al. (34)	Biopsies	Mild - severe	Cyclin D1	No staining in ASM layer
Trian et al. (33)	<i>In vitro</i>	Severe, persistent	Cell counts, BrdU	Yes
Kaur et al. (19)	<i>In vitro</i>	Mild - severe	Cell counts, TTI and CSFE	No
Benayoun et al. (2)	Biopsies	Mild - severe	Ki67	No staining in ASM layer
Johnson et al. (17)	<i>In vitro</i>	Mild- severe	Cell counts, TTI, DNA analysis	Yes

369
370 Abbreviations: ASM = airway smooth muscle; BrdU = 5-bromo-2'-deoxyuridine; CSFE =
371 carboxyfluorescein succinimidyl ester; PM = post-mortem; TTI = tritiated thymidine incorporation.
372

373 **Figure 1**

374 A. B.
375

376 Micrographs (x1000) from a case of nonfatal asthma, labelled with proliferating cell nuclear antigen
377 (PCNA) and counter-stained with haematoxylin. A) PCNA⁺ smooth muscle cells positive (solid
378 arrow) or PCNA⁻ cells (open arrow) can be seen in the airway smooth muscle layer. B.) As
379 expected, positive staining for PCNA is also seen in the airway epithelium (solid arrow).

380
381

382 **Figure 2**

383
384 Vertical dot plot showing the mean area density of proliferating airway smooth muscle cells
385 (cells/mm² x10³) in small (<6mm, basement membrane perimeter) and large (>6mm basement
386 membrane perimeter) airways. The horizontal line represents the medium group value.
387 Abbreviations: ASM = airway smooth muscle; Pbm = basement membrane perimeter; PCNA =
388 proliferating cell nuclear antigen.

389
390
391

392 **Figure 3**

393 Vertical dot plot showing the mean % PCNA positive airway smooth muscle cells of the total

394 number of airway smooth muscle cells, in small (<6mm, basement membrane perimeter) and large

395 (>6mm basement membrane perimeter) airways. The horizontal line represents the mean group

396 value. Abbreviations: ASM = airway smooth muscle; Pbm = basement membrane perimeter; PCNA

397 = proliferating cell nuclear antigen.

398

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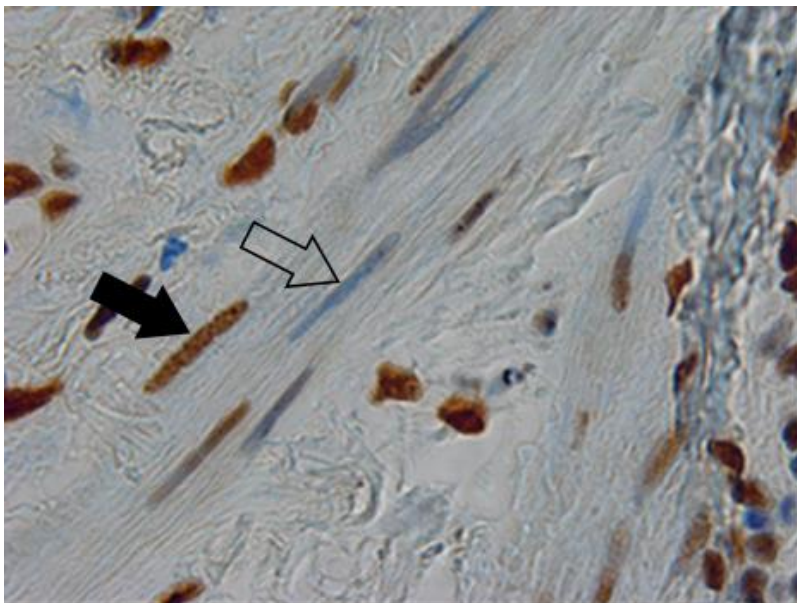
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B.

