

Estimating Airway Smooth Muscle Cell Volume and Number in Airway Sections

Sources of Variability

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Abstract

Hypertrophy and hyperplasia of airway smooth muscle (ASM) cells are features of asthma that can be assessed in airway transverse sections using stereologically derived parameters. However, little is known about the variability of these parameters within and between airways. The aim of the present study was to assess sources of variation in the measurement of the area of the ASM layer (A_{ASM}), and the volume fraction of ASM cells (V_{VASM}) and numerical density of ASM cells within that layer. A_{ASM} increased by up to 12% in 4- μ m sections, and 28% in 30- μ m sections, compared with 0.5- μ m sections. A_{ASM} was greater ($P < 0.05$) in large than in small airways, and varied by up to 28% along segments of large airways. Numerical density of ASM cell estimates around the airway circumference varied by less than 10% if 40 random high-power fields were sampled. V_{VASM} was most accurately estimated on 0.5- μ m, rather than 4- or 30- μ m sections, and was less ($P < 0.05$) in large than in small airways. The coefficients of variation for V_{VASM} were less than 10% along airway segments. We found that variation of parameters used to estimate ASM cell number or size could be minimized with adequate sampling frequency around or along airway segments. Section

thickness was positively related to the measured area of ASM on transverse airway sections. Thin (0.5- μ m) sections should be used to estimate tissue volume fractions, which vary little within and between airways of similar size. Airway size contributes most to the variation in estimating parameters of the ASM layer.

Keywords: airway smooth muscle; morphometry; stereology; variability

Clinical Relevance

Stereology of airway smooth muscle in airway disease has become the standard for accurate assessment of cell hyperplasia and hypertrophy. Currently, there are no data addressing the sources of variation (such as sampling site and effects of airway size) in these estimates. This study demonstrates potential sources of measurement error and how to avoid them when estimating smooth muscle cell volume and number in intact airways.

The increased thickness of the airway smooth muscle (ASM) layer in patients with asthma is related to asthma severity (1), and is associated with airway hyperresponsiveness (2). The ASM layer in a segment of airway can be thought of as a sleeve within the airway wall. The ASM layer is composed of ASM cells, extracellular matrix (ECM), and occasional blood vessels and inflammatory cells, all of which contribute to volume. Increased thickness of the ASM layer may be due to

increased numbers of ASM cells (hyperplasia) (3, 4) and/or increased size of ASM cells (hypertrophy) (5, 6), or to an increase in the volume of other elements, such as the ECM. Because different pathological processes may contribute to hypertrophy or hyperplasia of ASM cells in airway diseases (7), it has become increasingly important to measure the size and number of ASM cells in intact airways. However, little is known about the sources of variation in these measurements.

The number and size of ASM cells within the ASM layer can be estimated knowing the volume of the ASM layer per unit length, the numerical density (N_V) of ASM cells and the volume fraction of actual smooth muscle (V_{VASM}), excluding the ECM (V_{VECM}) and other elements within the ASM layer (V_{VOther}) (Figure 1). The volume of the ASM layer in a length of airway is given by the area of the ASM layer (A_{ASM}) seen on a sampled transverse section multiplied by length. N_V can be

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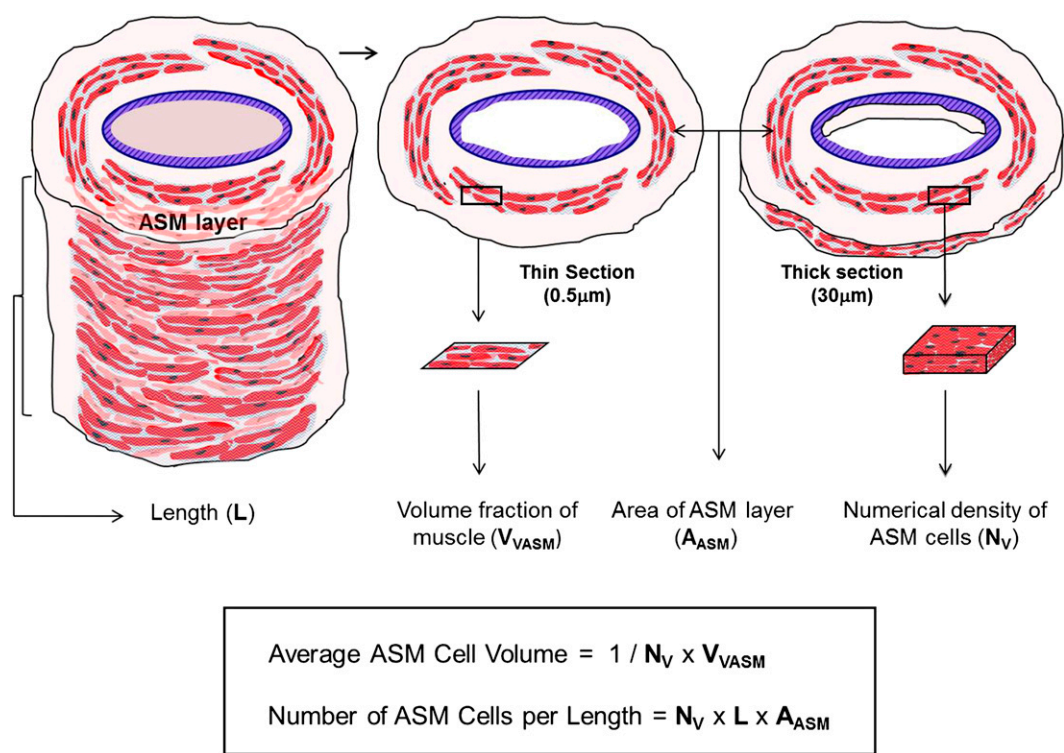


Figure 1. Schematic of a length of airway with the airway smooth muscle (ASM) layer. To estimate ASM cell size and number, thick and thin transverse sections of the airway were cut and sampled to derive the volume fractions of actual smooth muscle within the ASM layer (V_{VASM}) and the numerical density of ASM cells (N_V), respectively. Knowing the cross-sectional area of the ASM layer (A_{ASM}), the number of ASM cells per unit length of airway, and the average ASM cell volume can be estimated.

estimated by sampling a known volume of the ASM layer using the optical dissector approach (8, 9) on “thick” sections (30 μm). This accommodates efficiency of sampling and ensures sufficient counting events (ASM cell nuclei). As the ASM layer contains smooth muscle, ECM, and other elements, and we require only the volume of the smooth muscle, V_{VASM} must be calculated to avoid overestimating the average ASM cell volume. Estimating the volume fractions of various elements within the ASM layer is problematic, due to the overlap of ill-defined tissues within

a translucent volume (10). To overcome this problem, very thin sections must be used, although the optimal practical thickness is unknown. ASM parameters may vary within airway sections, along the length of airway segments, and between airways of different sizes. In this study, we examined, in large and small airways, variation of tissue components between sections along an airway segment, the effect of the number and location of sampling sites on estimates of N_V , and the effect of tissue section thickness on estimates of V_{VASM} .

Materials and Methods

Subjects

Archival tissue blocks of large and small airways (11) from 32 subjects (Table 1) were examined. This study was approved by the Human Research Ethics Committee of Sir Charles Gairdner Hospital (Nedlands, WA, Australia). Subjects were categorized as control subjects or those with nonfatal asthma or fatal asthma. One subject without available clinical data was categorized as “unclassified.” Details on tissue preparation and statistical analysis are in the online supplement.

Table 1: Subject Characteristics

Subject Group	Sex (M/F)	Age (yr)	Weight (kg)	Height (cm)	Smoking (Y/N/Ex/U)	Pbm (mm)
Control (n = 12)	11/1	27 ± 11	79 ± 20	172 ± 7	2/2/1/7	10.7 ± 8.4
Nonfatal asthma (n = 8)	2/6	30 ± 10	74 ± 33	159 ± 11	4/1/0/3	11.4 ± 10.0
Fatal asthma (n = 11)	7/4	49 ± 20	65 ± 16	171 ± 11	2/2/4/3	8.8 ± 7.1
Unclassified (n = 1)	—	—	—	—	—	11.1 ± 6.9

Definition of abbreviations: Ex, ex-smokers; F, female; M, male; N, nonsmokers; Pbm, perimeter of basement membrane; U, unknown smoking status; Y, current smokers. Not all subjects were used for all analyses.

Measurement of ASM Parameters and Variability

A_{ASM} . The total A_{ASM} , delineated by the outline of the ASM bundles around the airway wall, was measured ($\times 40$). The perimeter of the basement membrane (Pbm) was used to indicate airway size. The effect of section thickness on A_{ASM} was assessed in a large airway (Pbm > 10 mm) from nine subjects. Consecutive 0.5-, 4-, or 30- μ m sections were stained with hematoxylin and eosin. Variability of A_{ASM} (and other parameters [see subsequent text]) along the length of airway segments was assessed in one large and one small airway each from three subjects (control, asthma, and unclassified). From a random starting point, a 1-mm length of airway was sectioned (0.5 μ m for up to 10 μ m, plus three 30- μ m sections), every 100 μ m until tissue exhaustion. The effects of airway size (Pbm, 2–40 mm) on A_{ASM} were compared in at least 5 airways from each of 12 subjects.

N_V of ASM cells. N_V was estimated in randomly selected high-power fields ($\times 1,000$) on 30- μ m sections (hematoxylin) using the optical dissector approach (Figure 2) (10). The effect of numbers of samples from a site in the airway wall was assessed in five subjects. The running mean of N_V was determined in these sections by calculating the average N_V for every two additional random high-power fields until a maximum of 60 fields were sampled. The effect of sampling within sections was assessed in five subjects by estimating

N_V at four points around the airway circumference. These values were compared with the mean N_V obtained for the whole smooth muscle layer. The variability of N_V along airway segments and the effects of airway size were assessed as for A_{ASM} .

V_{VASM} . The effect of section thickness on estimated V_{VASM} was assessed on tissue sections of 0.5-, 4-, and 30- μ m thickness using point counting at $\times 1,000$ magnification (Masson's trichrome technique, Figure 3A). Sections of one large and one small airway each from two subjects were used. On three separate counts on each section, the ASM layer was systematically and randomly sampled around each transverse section until 200 points or more falling on ASM, ECM, or "Other" (Figure 3B) were counted. "Other" includes space due to fixation artifact, inflammatory cells, and blood vessels. For example, for the estimation of V_{VASM} , all points were scored either as "ASM" or "not-ASM." This process was repeated on the same sections for ECM and Other. If no overlap was present, the sum of the separately estimated fractions should equal 1. Variability of V_{VASM} along airway segments and the effects of airway size were assessed as for A_{ASM} .

Results

A_{ASM}

Compared with 0.5- μ m sections, the estimated A_{ASM} was increased by an average of 1.6 and 12.1% in 4- and 30- μ m

sections, respectively, in control subjects, and by 12.2 and 28.8% in 4- and 30- μ m sections, respectively, in subjects with fatal asthma (Figure 4). The mean differences between 0.5- and 30- μ m sections of the same large airways were 0.13 mm² for control subjects and 0.29 mm² for subjects with fatal asthma. The coefficient of variation (CV) for A_{ASM} measured on 30- μ m sections along airway segments ranged from 9.1 to 27.7%, and was not related to the absolute value (Table 2). The mean differences between 30- μ m sections were 0.12, 0.10, and 0.51 mm² for each of the control subjects and those with asthma and who were unclassified, equating to 13, 9, and 14% of the mean absolute values, respectively. A_{ASM} increased significantly with airway size in all subject groups (Table 3).

N_V

The variability of the running mean of N_V within sections decreased with increasing numbers of sampled high-power fields, and was less than 10% when 40 or more fields were sampled (Figure 5). This was independent of the sampling site around the airway circumference. The variability of N_V within (or along) airway segments was less in small airways than large airways, but similar across subjects. Thus, for control subjects, the between-section CV was 19.4% for small airways and 26.7% for large airways (Table 2). The between-airway (from the same person) CV for control subjects was 16.3% in the small airway and

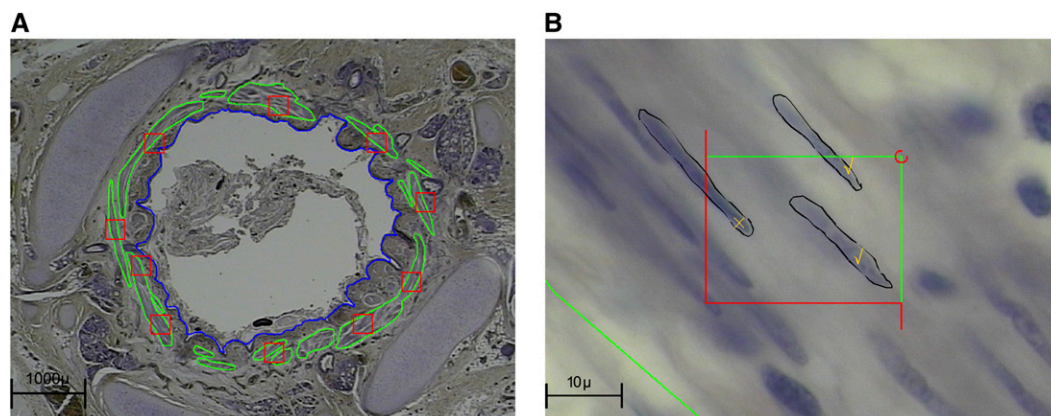


Figure 2. Estimation of ASM cell numerical density on a 30- μ m transverse airway section stained with hematoxylin. (A) Low power ($\times 10$) showing the basement membrane perimeter (blue line) and ASM layer (outlined in green). Software-generated random samples (red boxes) of the total (A_{ASM}) are shown. Scale bar, 1,000 μ m. (B) Sampled region ($\times 1,000$) of an ASM bundle. The green diagonal line indicates the margin of ASM bundle defined at low power. Cell nuclei within the counting frame of the optical dissector, or crossing the green inclusion lines, were counted (ticks) when they first came into focus as the focal plane was moved down through the depth of the optical dissector. Cell nuclei touching or crossing the red exclusion lines at any depth were not counted (cross). Scale bar, 10 μ m.

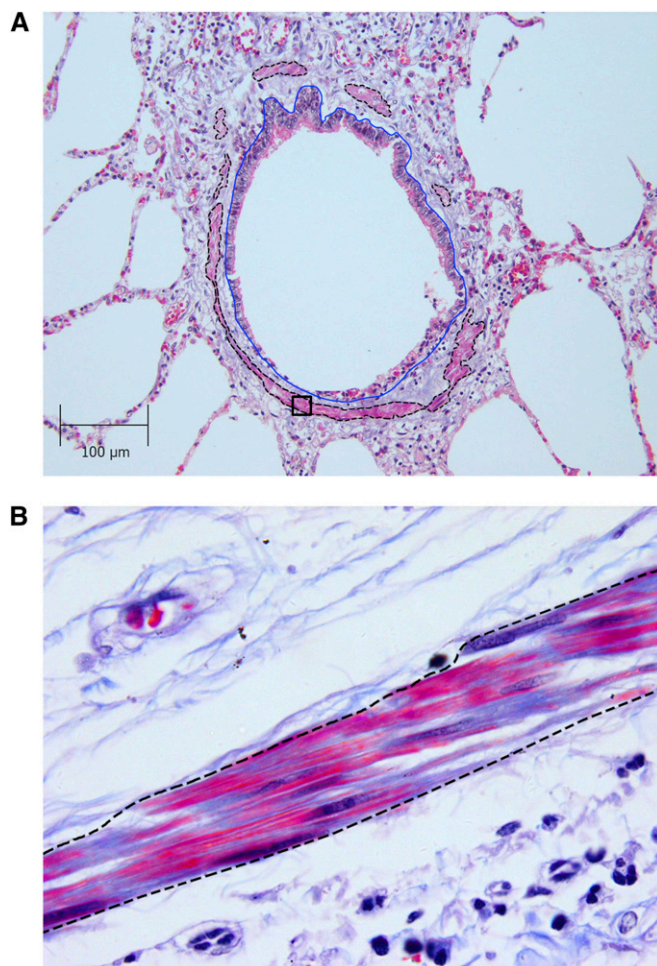


Figure 3. Estimation of tissue volume fractions on a 0.5- μ m transverse airway section stained using the Masson's trichrome technique. (A) Smooth muscle bundles (dashed outline) and perimeter of the basement membrane (blue) at low power ($\times 20$). Regions of smooth muscle were magnified for point counting (box). (B) Region of an ASM bundle (dashed outline) within the smooth muscle layer at high power ($\times 1,000$). A point grid was projected onto smooth muscle bundle to estimate the volume fractions of ASM cells (red), extracellular matrix (blue), and "Other" (white).

21.8% in the large airways (data not shown), and the between-subject CV was 17.2% in small airways and 23.0% in large airways (estimated from Table 3). The same patterns were seen in subjects with fatal asthma. The absolute values of N_V were not significantly different between airway sizes (Table 3).

V_{VASM} and V_{VECM}

The separately estimated fractions of V_{VASM} and V_{VECM} increased with section thickness, whereas V_{VOther} (mainly space) decreased. The sum of these separately estimated tissue fractions ($V_{VASM} + V_{VECM} + V_{VOther}$) increased from 1.08 and 1.09 (control and fatal asthma, respectively) in 0.5- μ m sections to 1.09 and 1.19 in 4- μ m sections and 1.26 and 1.22 in 30- μ m

sections (Table 4). The variation of V_{VASM} along airway segments was less than 10% in all airways (Table 2). V_{VASM} tended to decrease with increased airway size, and was significantly decreased in the large airways compared with small airways in the control subjects (Table 3).

Discussion

To understand the mechanisms of remodeling of the ASM layer in asthma and other airway diseases, it is necessary to estimate the relative contributions of ASM cell number and size and deposition of ECM. To estimate these parameters within the intact airway wall requires knowledge of the volume of the ASM layer, the volume

fractions of ASM and ECM (and other elements), and the N_V of ASM cells. We found that estimated A_{ASM} was affected by section thickness, most notably in 30- μ m sections in airways in which the A_{ASM} was increased, and we noted that the A_{ASM} varied as much as 30% along large airway segments. N_V was best estimated using 40 random high-power fields, and varied little around airway segments, and by less than 22% between airways in the same person. We also found that volume fractions of tissue components were optimally estimated on 0.5- μ m sections and changed little around or along the length of an airway.

The ASM layer forms an irregular sleeve around airways, made up of bands of muscle, roughly perpendicular to the length of the airway (12), and it varies in thickness along large airways (3, 13), as we found in the present study. To measure the A_{ASM} , hematoxylin-stained section were used. This stain is adequate for the accurate measurement of the A_{ASM} , as we found no differences in the measured A_{ASM} in sections from large and small airways from subjects with and without asthma stained with hematoxylin or stained using an antibody to a specific ASM protein (α -smooth muscle actin—see Table E1 in online supplement). The variation of the N_V of ASM cells was similar between sections, between airways, and between subjects. The tissue fractions within the ASM layer varied little along airways, and the variability of V_{VASM} (5–9%) and V_{VECM} (12–29%) observed along airways were similar to estimates of airway dimensions (1–7%) and inflammatory cell counts (11–34%) reported previously (14). Using standard formulae, such as that of Dobson (15) for normally distributed continuous data, we can estimate the number of subjects required to detect differences between subject groups by knowing the variability of these measures.

The CV of the A_{ASM} measured on 30- μ m sections was found to vary between 9 and 30% along segments of large airways, and was independent of the absolute value. The variation in A_{ASM} between 30- μ m sections along the same airway observed in the present study was similar to those observed in 4- μ m sections by Heard and Hossain (3). In the present study, variation of estimated A_{ASM} due to different section thickness was also observed. A_{ASM} was seen to increase more in thicker

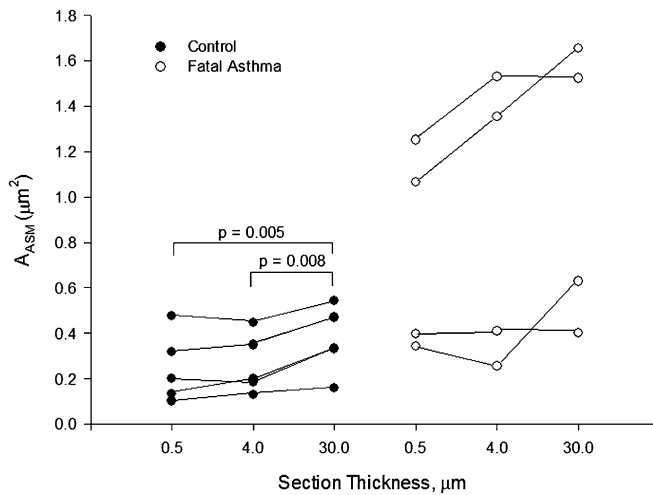


Figure 4. Measured cross-sectional area of ASM (A_{ASM}) in 0.5-, 4-, and 30- μ m sections of large airways from five control subjects and four subjects with fatal asthma.

sections from subjects with fatal asthma (28.8% increase) compared with thicker sections from control subjects (12.1% increase; Figure 4). This results from the increased variation in the absolute thickness of the ASM layer (rather than that relative to the mean thickness, the CV as in Table 2) in subjects with asthma compared with subjects without asthma. The observed increase in the estimated thickness of A_{ASM}

is due to increasing overlap with increasing section thickness (*see* also subsequent text). Therefore, both the site along an airway segment and the section thickness will contribute to the variation in estimated A_{ASM} obtained from a single airway section. We suggest that the use of 30- μ m sections will minimize variation due to the differences observed in A_{ASM} over very short lengths of airway within subjects,

although this may lead to a very small overestimation of average A_{ASM} compared with 0.5- μ m sections. This bias is very small relative to the differences observed between control subjects and subjects with asthma.

In thick sections, tissue elements within the ASM layer overlap and, in the case of the ill-defined ECM and spaces due to artifact or edema, cannot easily be distinguished. In very thin sections, overlap of tissues is minimized, and the area (and therefore volume) fractions of these structures can be estimated. When we estimated the tissue fractions separately on increasingly thick sections, the effects of tissue overlap were clearly observed. The increased estimated V_{VASM} that we observed in relation to section thickness would result in overestimation of volume fractions by roughly 25 and 20% in 30- and 4- μ m sections, respectively. As the tissue thickness increased, the separately estimated fractions of both ASM and ECM increased, due to tissue overlap. The fraction of “other,” however, decreased, as this fraction comprised mainly space, which diminishes due to overlap of ECM and ASM as the section thickness increases.

Mean ASM cell volume in intact tissues can be estimated using the inverse of the

Table 2: Variability of Dimensions and Components of the Airway Smooth Muscle Layer in Transverse Sections along 1-mm Lengths of Small and Large Airways

Subject	Pbm (mm)	A_{ASM} (mm ²)	N_V Cells/ μ m ³ $\times 10^{-4}$	V_{VASM}
Small airways ($n = 26$ sections)				
Measured Values (mean $\pm$ SD)				
C	3.6 $\pm$ 0.4	0.03 $\pm$ 0.01*	1.91 $\pm$ 0.37*	0.78 $\pm$ 0.04*
FA	5.0 $\pm$ 0.3	0.10 $\pm$ 0.02*	1.72 $\pm$ 0.27	0.71 $\pm$ 0.04 [†]
U	4.1 $\pm$ 0.4	0.10 $\pm$ 0.02*	1.79 $\pm$ 0.27*	0.74 $\pm$ 0.06*
CV, %				
C	11.2	25.6	19.4	4.7
FA	6.4	18.5	15.6	5.8
U	10.7	15.5	15.3	8.1
Large airways ($n = 27$ sections)				
Measured Values (mean $\pm$ SD)				
C	16.6 $\pm$ 0.9	0.90 $\pm$ 0.17	1.45 $\pm$ 0.39	0.69 $\pm$ 0.06
FA	12.2 $\pm$ 0.6	1.15 $\pm$ 0.10	1.74 $\pm$ 0.26	0.69 $\pm$ 0.05
U	17.5 $\pm$ 1.0	2.00 $\pm$ 0.55 ^{†,‡}	1.40 $\pm$ 0.32	0.67 $\pm$ 0.04
CV, %				
C	5.4	19.4	26.7	9.3
FA	3.9	9.1	15.2	7.9
U	5.5	27.7	22.8	5.7

Definition of abbreviations: A_{ASM} , area of airway smooth muscle; C, control; CV, coefficient of variation; FA, fatal asthma; N_V , ASM cell numerical density; Pbm, perimeter of basement membrane; U, unclassified; V_{VASM} , volume fraction of ASM cells.

* $P \leq 0.05$ versus large airways.

[†] $P < 0.02$ versus C.

[‡] $P < 0.02$ versus FA.

Table 3: Effect of Airway Size on Airway Smooth Muscle Area, Density of Airway Smooth Muscle Cells, and Volume Fraction of ASM in Control Subjects ($n = 4$) and Those with Nonfatal Asthma ($n = 4$) and Fatal Asthma ($n = 4$)

Case	Pbm (mm)	A_{ASM} (mm ²)	N_V cells/ $\mu\text{m}^3 \times 10^{-4}$	V_{VASM}
Small ($n = 22$ airways)				
C	3.5 ± 1.3	0.09 ± 0.05	1.84 ± 0.32	0.67 ± 0.07
NFA	3.1 ± 0.6	0.08 ± 0.03	2.08 ± 0.36	0.68 ± 0.06
FA	2.9 ± 1.1	0.11 ± 0.10	1.92 ± 0.53	0.69 ± 0.07
All	3.2 ± 1.0	0.09 ± 0.06	1.95 ± 0.31	0.68 ± 0.07
Medium ($n = 23$ airways)				
C	6.7 ± 1.0	0.23 ± 0.11	1.46 ± 0.20	0.65 ± 0.06
NFA	7.1 ± 1.6	$0.36 \pm 0.18^*$	1.79 ± 0.46	0.62 ± 0.06
FA	6.3 ± 1.6	0.53 ± 0.37	$2.11 \pm 0.48^{\dagger}$	0.69 ± 0.07
All	6.7 ± 1.4	0.38 ± 0.27	1.79 ± 0.48	0.65 ± 0.06
Large ($n = 24$ airways)				
C	19.1 ± 11.0	$1.24 \pm 0.86^{*,\dagger}$	1.70 ± 0.39	$0.57 \pm 0.09^*$
NFA	20.7 ± 9.8	$0.98 \pm 0.94^*$	1.69 ± 0.29	0.66 ± 0.11
FA	20.2 ± 10.1	$2.02 \pm 1.37^*$	2.09 ± 0.90	0.65 ± 0.16
All	20.0 ± 9.9	1.29 ± 1.04	1.78 ± 0.48	0.62 ± 0.12

Definition of abbreviations: A_{ASM} , area of airway smooth muscle; C, control; FA, fatal asthma; NFA, nonfatal asthma; N_V , ASM cell numerical density; Pbm, perimeter of basement membrane; V_{VASM} , volume fraction of ASM cells.

Small airways: Pbm, <5 mm; medium airways: Pbm, 5–10 mm; large airways: Pbm, >10 mm.

* $P < 0.05$ versus small airways.

$^{\dagger}P = 0.05$ versus medium airways.

N_V in that tissue (Figure 1). In previous studies (3–5), the volume portion of N_V has included ASM, ECM, and other elements. This may have resulted in overestimation of ASM cell size. The study of Ebina and colleagues (5), which used stereological techniques similar to those in the present study, but did not correct for ECM, showed a mean ASM cell volume in large airways of approximately $4,500 \mu\text{m}^3$ in control subjects. This value is considerably higher than that observed ($2,757 \mu\text{m}^3$ in control

subjects) where ECM and other components have been excluded from volume of the ASM layer used to estimate mean ASM cell volume (9). ASM cell volume, estimated from biopsy samples of proximal airways (4, 6), and not correcting for ECM, are lower than estimation from postmortem tissue sections ranging from approximately $2,000$ to $2,700 \mu\text{m}^3$. It is possible that biopsies yield lower values for ASM cell volume, due to tissue preparation artifacts or differences between live and

post-mortem specimens. Systematic differences in the components of the ASM layer between disease and control subjects could also result in an overestimation of the degree of hypertrophy in disease.

Variability of airway components between airways of different size is important, because examination of airway pathology in most patients is necessarily confined to biopsy of the large (central) airways. We found that V_{VASM} decreased as airway size increased, and this trend was significant in control airways, although more variable in cases where the ASM layer was thicker (fatal asthma). The same trend was observed in a previous study (9); however, a significant difference was only observed in the large airways of subjects with fatal asthma in that study. This may be a reflection of the comparatively small numbers of cases examined in the present study. The observed effects of airway size likely reflect the relative increase in non-smooth muscle elements in the ASM layer in large airways, and suggest that predicting remodeling changes in the small airways on the basis of findings from biopsies of the large airways should be undertaken with caution.

Although the fixation in formalin and embedding in paraffin used in the present study is excellent for preservation of

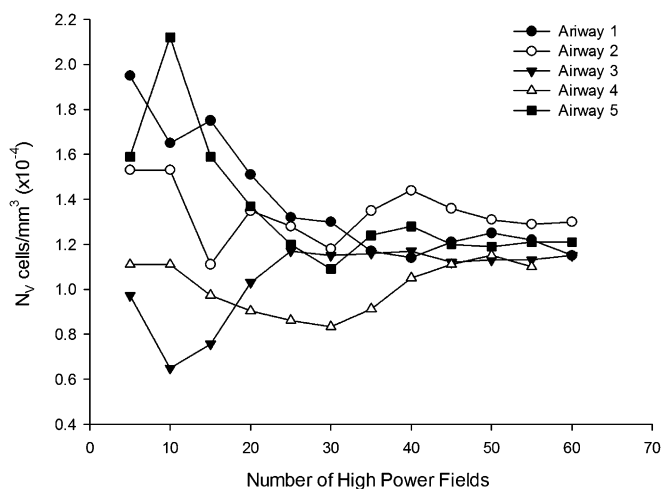


Figure 5. Running means of ASM cell numerical density (N_V) for five airways. N_V was plotted for every additional five high-power fields.

Table 4: The Volume Fractions of Smooth Muscle, Extracellular Matrix, and Other Elements in Large Transverse Airways Sections of 0.5-, 4-, and 30- μm Thickness from a Control Subject and a Subject with Fatal Asthma

	0.5 μm		4 μm		30 μm	
	C	FA	C	FA	C	FA
V_{VASM}	0.52	0.58	0.61	0.69	0.77	0.80
V_{VECM}	0.28	0.28	0.33	0.33	0.39	0.33
V_{VOther}	0.28	0.23	0.16	0.17	0.10	0.09
Total	1.08	1.09	1.09	1.19	1.26	1.22

Definition of abbreviations: C, control; FA, fatal asthma; V_{VASM} , volume fraction of ASM cells; V_{VECM} , volume fraction of extracellular matrix; V_{VOther} , volume fraction of other elements, including space between cells and inflammatory cells.

structure, it results in unpredictable tissue shrinkage (16). Although the degree of tissue shrinkage was not assessed in the present study, it is unlikely to have affected the within-airway comparisons that we have made, and will not invalidate our conclusions from these data. However, shrinkage increases the potential for variation (more noise) or bias (less accuracy) when comparing tissues from different lungs. Another technical factor is the ability of the Masson's trichrome

technique to stain all ECM elements within tissue sections. As far as we are aware, there are no studies to show whether this or any other staining technique includes all ECM proteins identified within the ASM layer—fibronectin, versican, biglycan, decorin, lumican, tenascin, as well as collagen and elastin. To determine whether the Masson's technique does stain all ECM would require quantification of the volume fractions of each individual ECM protein on sequential thin (0.5- μm) airway

sections. The volume fractions of each ECM component would then need to be totaled and compared with an airway section stained with the Masson's trichrome technique. This is currently an area of further research.

In summary, the sources of variation that we observed can have significant effects on the estimation of ASM cell volume and number, and will confound comparisons between individuals. After adequate sampling, the most significant source of variation is airway size for the estimation of V_{VASM} . We recommend that 40 or more high-power fields be used to estimate ASM cell N_V , that 0.5- μm sections are optimal for estimating V_{VASM} , and that thicker sections (e.g., 30 μm) be used to compare the thickness of the ASM layer. Implementing these strategies should result in accurate estimations of ASM cell parameters when assessing the effects of diseases such as asthma and chronic obstructive pulmonary disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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ESTIMATING AIRWAY SMOOTH MUSCLE CELL VOLUME AND NUMBER IN
AIRWAY SECTIONS: SOURCES OF VARIABILITY

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Online Data Supplement

Tissue preparation and measurement of airway dimensions

Lungs were fixed by inflation with formalin at 25cm H₂O. Blocks of airways from central to sub-segmental level were embedded in paraffin. Transverse sections of 30µm, 4µm or 0.5µm thickness were cut as required. Measurements were made using stereological software (newCAST, Denmark).

Statistical Analysis

The effects of section thickness and airway size on A_{ASM} , N_V and V_{VASM} were assessed using ANOVA. Variability along airway segments was expressed as the coefficient of variation ($CV=SD/mean \times 100\%$). Normality was tested using the Shapiro-Wilk test with an equal variance test performed if data were normally distributed or non-parametric post-hoc tests of significance were used if appropriate. Parameters from subjects with multiple airways were assessed using subject means. Sources of variability were compared between subject groups (control, nonfatal and fatal asthma, and unclassified) as appropriate. The effects of airway size (small airways $P_{bm} < 5mm$, medium airways $P_{bm} 5-10mm$, large airways, $P_{bm} > 10mm$) were examined within subject groups. Tabulated results are displayed as mean $\pm$ standard deviation.

Table E1: ASM Areas (mm²) in Actin- and Haematoxylin-stained Sections.

Stain	Large	Small	Control	Asthma
Actin	0.66 ± 0.30	0.07 ± 0.09	0.22 ± 0.22	0.50 ± 0.46
Haematoxylin	0.64 ± 0.30	0.06 ± 0.08	0.22 ± 0.22	0.49 ± 0.46

Mean ± standard deviation