

Growth of the airway smooth muscle layer from late gestation to childhood is mediated initially by hypertrophy and subsequently hyperplasia

Kimberley C. W. Wang^{1,2}  | Graham M. Donovan³ | Sejal Saglani⁴ | Thais Mauad⁵ | Alan L. James^{6,7}  | John G. Elliot^{1,6}  | Peter B. Noble¹ 

¹School of Human Sciences, The University of Western Australia, Crawley, Western Australia, Australia

²Teleton Kids Institute, The University of Western Australia, Nedlands, Western Australia, Australia

³Department of Mathematics, University of Auckland, Auckland, New Zealand

⁴National Heart and Lung Institute, Imperial College London, London, UK

⁵Department of Pathology, University of São Paulo, São Paulo, Brazil

⁶Department of Pulmonary Physiology and Sleep Medicine, West Australian Sleep Disorders Research Institute, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia

⁷Medical School, The University of Western Australia, Nedlands, Western Australia, Australia

Correspondence

Kimberley C. W. Wang
Email: kimberley.wang@uwa.edu.au

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Abstract

Background and objective: The airway smooth muscle (ASM) layer thickens during development. Identifying the mechanism(s) for normal structural maturation of the ASM reveals pathways susceptible to disease processes. This study characterized thickening of the ASM layer from foetal life to childhood and elucidated the underlying mechanism in terms of hypertrophy, hyperplasia and extracellular matrix (ECM) deposition.

Methods: Airways from post-mortem cases were examined from seven different age groups: 22–24 weeks gestation, 25–31 weeks gestation, term (37–41 weeks gestation), <0.5 year, 0.5–1 year, 2–5 years and 6–10 years. The ASM layer area (thickness), the number and size of ASM cells and the volume fraction of ECM were assessed by planimetry and stereology.

Results: From late gestation to the first year of life, normalized ASM thickness more than doubled as a result of ASM hypertrophy. Thereafter, until childhood, the ASM layer grew in proportion to airway size, which was mediated by ASM hyperplasia. Hypertrophy and hyperplasia of ASM were accompanied by a proportional change in ECM such that the broad composition of the ASM layer was constant across age groups.

Conclusion: These data suggest that the mechanisms of ASM growth from late gestation to childhood are temporally decoupled, with early hypertrophy and subsequent proliferation. We speculate that the developing airway is highly susceptible to ASM thickening in the first year of life and that the timing of an adverse event will determine structural phenotype.

KEYWORDS

airway smooth muscle, asthma, extracellular matrix, hyperplasia, hypertrophy, ontogeny, remodelling

INTRODUCTION

There is little doubt regarding the adverse consequences of airway smooth muscle (ASM) remodelling in asthma and that this pathology occurs early in the clinical course of disease. Increased thickness of the ASM layer in asthma

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exaggerates airway narrowing to contractile provocation¹ and is associated with more severe disease.² Children with asthma already exhibit a greater proportion of ASM in airway biopsies,³ and similar expansion of the ASM is observed in pre-school children who go on to develop asthma at school age,⁴ that is, remodelling seems to precede diagnosis. In adult life, thickness of the ASM layer does not change and is unaffected by the duration of disease.² If we assume a tight coupling between airway structure–function, there is even evidence of airway pathology from the first year of life. Flow impairment and increased airway reactivity in infancy predict asthma in later life.⁵

Much of our understanding regarding the role of ASM in asthma has been advanced through use of airway tissue acquired post-mortem or after lung resection surgery. Using such methods, we have shown that ASM thickening in asthma is due to both muscle hypertrophy and hyperplasia, and a proportionate increase in extracellular matrix (ECM).⁶ Whether the same structural changes account for normal developmental growth of the ASM layer is not known but is important to establish as disease processes may interfere and/or upregulate mechanisms controlling tissue expansion, thereby contributing to early airway pathology. There is also no reason to assume that muscle hypertrophy and hyperplasia, and ECM deposition within the ASM layer occur during the same periods of development and indeed the specific timing of any disruptive stimulus could contribute to known phenotypes of ASM remodelling. For instance, hypertrophy occurs in both non-fatal and fatal asthma, whereas hyperplasia is observed in the latter.⁶ On the other hand, fixed air-flow limitation seems to be mediated by a disproportionate increase in ECM within the ASM layer.⁷

The purpose of this study was to characterize normal mechanisms of ASM growth in terms of hypertrophy, hyperplasia and/or ECM deposition. Airway tissue from different regions of the lung was examined across seven different age groups, spanning late gestation to childhood. Findings demonstrate that the mechanism of ASM growth is due to early hypertrophy and late hyperplasia, with proportionate expansion of the ECM within the ASM layer.

METHODS

Study participants

Tissue is stored in the Airway Disease Biobank at Sir Charles Gairdner Hospital. Airways examined in the present study were sourced from post-mortem studies of asthma based in Perth (1), London (24), Sydney (13) and São Paulo (13). As shown in Table 1, airways from post-mortem cases were available from seven a priori classified age groups: 22–24 weeks gestational age (GA; $n = 8$), 25–31 weeks GA ($n = 8$), term (37–41 weeks GA; $n = 8$), <0.5 year old ($n = 8$), 0.5–1 year old ($n = 8$), 2–5 years old ($n = 8$) and 6–10 years old ($n = 3$). Information regarding the history of

SUMMARY AT A GLANCE

A period of rapid hypertrophic airway smooth muscle growth occurs in the first year of life, representing a critical window for disruption by disease processes and/or opportunity for clinical intervention.

asthma (and cause of death) was sought from next-of-kin, hospital files, coroner files, police reports and the subjects' usual medical practitioners. Reported causes of death varied (see Appendix S1 in the Supporting Information) and include still birth, respiratory distress, infection, tumours and trauma. Two cases of death were undetermined, and one case was not recorded. Subjects from 2 years old onwards had no history of asthma or other lung disease. The proportion of males and females was similar across age groups (chi-square analysis; $p = 0.6$).

Tissue preparation

For subjects up to 5 years of age, a random section of lung tissue was fixed and embedded in paraffin. Tissue preparation and subsequent measurement followed protocols optimized to reduce variability and error.⁸ In the 6–10 years group, airways were sampled according to a systematic design,⁹ and also fixed and embedded in paraffin. From each case, two sections of tissue were cut: a thin section (0.5 μm , Masson's trichrome technique) for estimation of tissue volume fractions (ECM or smooth muscle) within the ASM layer, and a thick section (30 μm , haematoxylin) to estimate ASM cell number and size.

Airway measurements

The median (interquartile range) number of visually transverse airway sections studied per case was 7 (6–10). The

TABLE 1 Group characteristics

Group	Age	Sex (M:F)
22–24 weeks GA	23 \pm 0.3 weeks GA	4:4
25–31 weeks GA	27 \pm 0.7 weeks GA	6:2
Term	40 \pm 0.5 weeks GA	4:4
<0.5 year	0.17 \pm 0.04 year	4:4
0.5–1 year	0.7 \pm 0.06 year	3:5
2–5 years	2.1 \pm 0.17 years	2:6
6–10 years	7.5 \pm 1.0 years	2:1

Note: Values are mean \pm SEM.

Abbreviations: F, female; GA, gestational age; M, male.

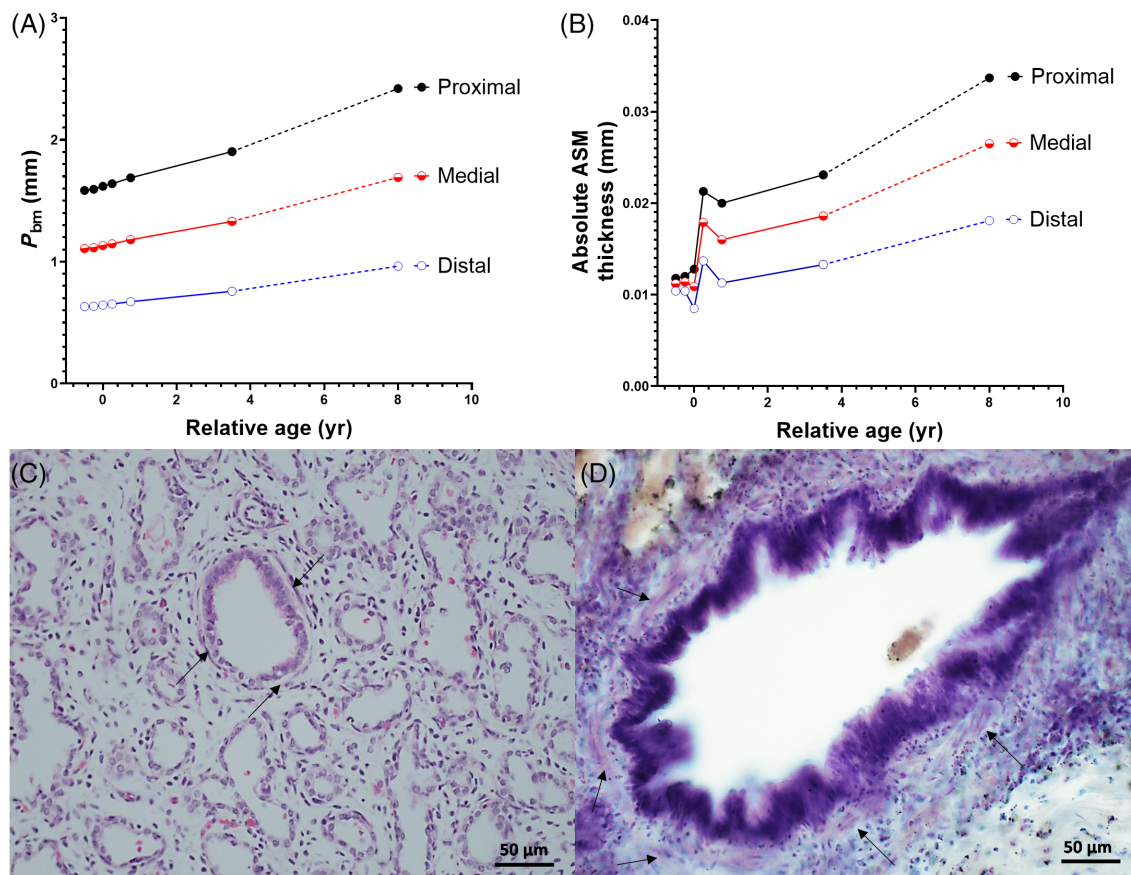


FIGURE 1 Predictive change in P_{bm} (A) and ASM thickness (B) with age and exemplar images (<24 weeks gestational age, C; 6–10 years, D). Arrows indicate ASM. Black circles and line, proximal airways; red circles and line, medial airways; blue circles and line, distal airways. The dashed lines indicate that predictions were based on limited data. ASM, airway smooth muscle; P_{bm} , perimeter of basement membrane; yr, year(s)

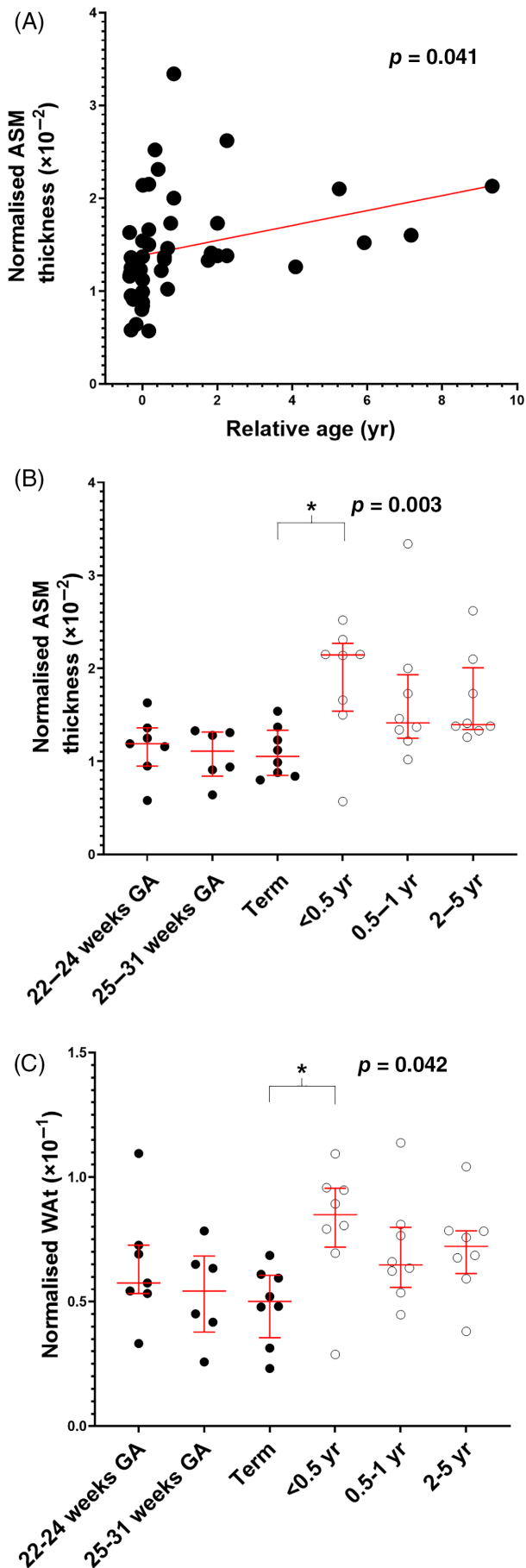
reticular basement membrane perimeter (P_{bm} , the index of airway size¹⁰) and area of the ASM layer (ASM_{area})⁶ were measured by planimetry on thick sections. Thickness of the ASM layer was calculated from ASM_{area} divided by P_{bm} . The same thick section was used to measure numerical density of ASM cells (N_V) using the optical disector approach ($\Sigma Q/(a \times h \times \Sigma P)$), where Q is the number of cells counted per disector, a and h are the area and height of the disector, respectively, and P is the number of disectors counted per section.¹¹ For the very smallest of airways (arbitrarily defined as <0.6 mm P_{bm}), all ASM cells within the layer were counted and N_V was calculated as: $\Sigma Q/(ASM_{area} \times h)$. As the depth of the thick section effectively equates to airway length, the total number of ASM cells per millimetre length of airway (N_L) was calculated as: $N_L = N_V$ (cells/mm³) \times ASM_{area} (mm²). An increase in N_L has previously been used to reflect hyperplasia in the context of asthma diagnosis.⁶ Mean ASM cell volume (V_C) was calculated as the inverse of N_V , corrected for V_{VASM} (see below): $V_C = 1/[N_V \times V_{VASM}]$.⁶

To assess volume fractions within the ASM layer, two airways per case were randomly selected. Volume fractions of ASM (V_{VASM}), ECM (V_{VECM}) and ‘Other’ (V_{VOTHER} , the space between tissues, non-muscle cells and vascular

structures) within the ASM layer were estimated by point counting.⁶

Statistical and data analyses

Primary outcomes were ASM thickness (ASM_{area}/P_{bm}), V_C , N_L , V_{VASM} , V_{VECM} and V_{VOTHER} . For parameters not dependent on airway size (V_C , V_{VASM} , V_{VECM} and V_{VOTHER}), a case mean was determined by averaging across all measured airways. As ASM thickness and N_L are dependent on airway size (P_{bm}), which varies with anatomical location and maturation, we used a method based on Pascoe et al.¹² to generate a representative and scaled airway for each case across all age groups: (1) ASM thickness and N_L were first shown to follow a power law = $c \times (P_{bm})^b$, where b describes how the parameter varies with P_{bm} and c the value at size $P_{bm} = 1$ mm (referred to as ‘normalized’ ASM thickness, N_L or WAt), and (2) to estimate absolute changes in ASM thickness and N_L , P_{bm} growth curves (Figure 1A) were established from computed tomography (CT)-derived tracheal lumen dimensions,^{13,14} assuming that intraparenchymal airways grow in proportion to the trachea. Growth curves (changes in P_{bm} from late gestation



to childhood) were generated for an arbitrary distal ($P_{bm} = 0.63$ mm), medial ($P_{bm} = 1.11$ mm) and proximal airway ($P_{bm} = 1.58$ mm) at 22–24 weeks GA to model changes across different airway sizes. Absolute ASM thickness and N_L could then be calculated from the normalized data (see ‘1’ above), multiplied by P_{bm} .

Based on data distribution, parameters were analysed using one-way ANOVA (Holm Sidak’s post hoc test) or Kruskal–Wallis one-way ANOVA on ranks (Dunn’s post hoc test) using SigmaPlot (version 13, Chicago, IL, USA). Prism (version 7, San Diego, CA, USA) was used for graphical analyses. Post hoc comparisons were compared with the term group, an appropriate reference for examining in utero and/or postnatal changes. Due to the lower sample size, the 6–10 age group was not included in any categorical statistical analysis. $p < 0.05$ was defined as statistical significance.

RESULTS

Figure 1B indicates predictive ASM growth from late gestation to childhood, which is supported by exemplar images (Figure 1C,D) chosen so that P_{bm} as a proportion of the trachea was matched across groups. Normalized ASM thickness was positively correlated with age ($p = 0.041$, $r = 0.297$; Figure 2A). When comparing the a priori classified age groups, much of the growth occurred up until <0.5 year age ($p = 0.003$; Figure 2B). Similar changes were observed in total wall thickness ($p = 0.042$; Figure 2C).

Growth of the ASM layer can initially be accounted for by hypertrophy ($p < 0.001$; Figure 3A). The V_C at 0.5–1 year was greater than term ($p = 0.005$). Changes in V_C likely caused a reduction in normalized N_L ($p = 0.003$; Figure 3B), that is, an increase in ASM cell size naturally reduces the number of cells that can be accommodated along a given mm length of airway (i.e., N_L). There was no increase in V_C after 0.5–1 year of age.

The V_{VASM} ($p = 0.158$) and V_{VECM} ($p = 0.150$) were not significantly different from pre- to postnatal life. Given that ASM thickness increases with age, constant volume fractions support a proportionate increase in the ECM and muscle within the ASM layer during maturation (Table 2). There was an overall main effect of age on V_{VOTHER} ($p = 0.007$; Table 2), but no differences between groups after post hoc analysis.

As indicated above, ASM hypertrophy directly impacts the measurement of N_L which we used to assess hyperplasia. To isolate hyperplasia opposed to hypertrophy (assuming a

FIGURE 2 Normalized ASM thickness plotted against continuous age (A) or presented in stratified age groups (B). Normalized total wall thickness presented in stratified age groups (C). Data are median (interquartile range). ASM, airway smooth muscle; GA, gestational age; WAT, total wall thickness; yr, year(s). *Significantly different from term ($p < 0.05$)

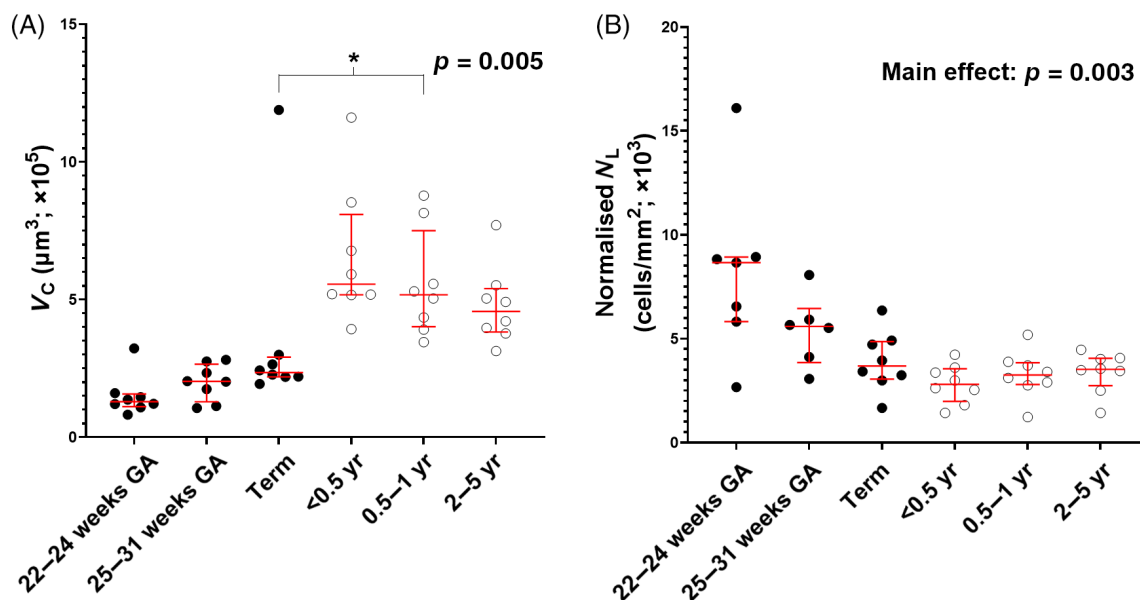


FIGURE 3 The ASM cell volume (A) and N_L (B) presented in stratified age groups. Data are median (interquartile range). ASM, airway smooth muscle; GA, gestational age; N_L , total number of ASM cells per millimetre length of airway; V_C , mean ASM cell volume; yr, year(s). *Significantly different from term ($p < 0.05$). 'Main effect' indicates an overall effect of age on N_L ; there were no differences after post hoc analysis

TABLE 2 Proportion of V_{VASM} , V_{VECM} and V_{VOTHER} within the ASM layer

Group	V_{VASM}	V_{VECM}	V_{VOTHER}^a
22–24 weeks GA	0.82 ± 0.08	0.11 ± 0.11	0.08 ± 0.10
25–31 weeks GA	0.84 ± 0.14	0.10 ± 0.07	0.07 ± 0.12
Term	0.83 ± 0.11	0.12 ± 0.08	0.06 ± 0.10
<0.5 year	0.84 ± 0.19	0.10 ± 0.11	0.07 ± 0.10
0.5–1 year	0.84 ± 0.19	0.08 ± 0.11	0.08 ± 0.12
2–5 years	0.87 ± 0.13	0.11 ± 0.08	0.02 ± 0.05

Note: Values are mean \pm SEM.

Abbreviations: ASM, airway smooth muscle; GA, gestational age; V_{VASM} , volume fraction of ASM; V_{VECM} , volume fraction of extracellular matrix; V_{VOTHER} , volume fraction of other.

^aSignificant main effect of age on V_{VOTHER} ($p < 0.05$). There was no difference after post hoc analysis.

proportionate increase in ECM as the muscle components expand), we modelled the effects of ASM thickening occurring solely due to hypertrophy (Figure 4). Hypertrophic growth of the ASM layer (dotted lines) was compared with the empirical data (solid/dashed lines). Results indicate that while ASM growth is entirely explained by ASM hypertrophy up and until 0.5 year of age, thereafter, it does not account for ASM thickening, and hyperplasia must be occurring until at least early childhood.

DISCUSSION

Age-dependent changes in airway and lung function are well established¹⁵ and naturally impact the onset and severity of respiratory disease. This study concerned normal growth of

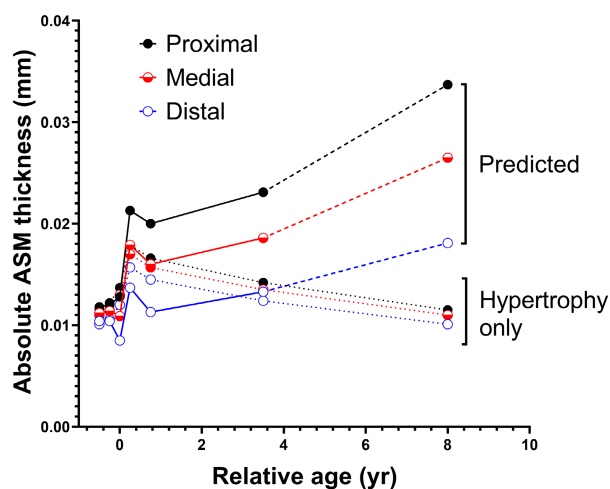


FIGURE 4 Modelled effects of ASM thickening occurring solely due to isotropic growth of ASM cells without hyperplasia (hypertrophy only; dotted lines) compared with predicted ASM growth (outputs are the same as those presented in Figure 1B). Hypertrophy cannot account for ASM growth beyond 0.5 year. Black circles and lines, proximal airways; red circles and lines, medial airways; blue circles and lines, distal airways. ASM, airway smooth muscle; yr, year(s)

the ASM layer, a structure that is prominent in asthma due to its increased thickness, promoting exaggerated constriction of the airway lumen.¹ Through morphological and stereological examination of human airway tissue, we show that the thickness of the ASM layer increases from late gestation to early childhood, in general agreement with previous findings,¹⁶ but importantly is initially due to hypertrophy and subsequently hyperplasia. Most notably, during the first year of life, there is rapid and

disproportionate ASM growth, marking a period for disease susceptibility and potentially therapeutic intervention.

Morphometric indices of the airway wall are dependent on both airway size and anatomical location (e.g., segmental or sub-segmental bronchus). In adult subjects, the conventional approach is to match airways based on P_{bm} , an accepted marker of airway size.¹⁰ In the context of an ontogeny study, it is perhaps more appropriate to match based on anatomical location, as P_{bm} will increase as the airway grows. Random sampling of airways (for most cases) meant that anatomical matching was not possible. Our approach was to establish a single representative airway for each human case based on the relationship between P_{bm} and ASM thickness as described by a power law. This regression analysis enabled us to scale each representative airway to a fixed arbitrary size of 1 mm P_{bm} and effectively remove the complication of airway size (referred to as normalized ASM thickness). The final step in the analysis was to estimate changes in intra-parenchymal airway size from late gestation to childhood through interpolation and extrapolation of the available CT-derived tracheal dimensions.^{13,14} These studies also support proportional growth across generations (our inherent assumption), at least for conducting sized airways.

Results demonstrate two clear phases of ASM growth. Immediately after birth for ~ 1 year, there is disproportionate thickening of the ASM layer (and also total airway wall) relative to airway size (P_{bm}), reflected by an increase in normalized ASM thickness, that is, the ASM layer grows more rapidly than the airway lumen dimension. Growth of the ASM layer during the first phase is entirely explained by hypertrophic growth (increased cell volume), as modelled in Figure 4. Under these circumstances, the parameter N_L becomes less informative and insensitive to hyperplasia as the number of cells that can be contained within a given length of airway is reduced as the volume of each cell unit expands, and hence N_L tends to fall with age. The second phase of ASM growth is marked by proportionate ASM growth that occurs without any further change in ASM cell volume, and therefore must be due to hyperplasia (assuming no shift in the proportion of ECM; see below). The increased number of ASM cells will be deposited radially as the circumference of the airway increases with age, and also transversely so that the normalized thickness of ASM remains constant.

When assessing expansion of the ASM layer, either in the context of normal or abnormal growth, changes in ECM opposed to ASM tissue require consideration. It is feasible that an increase in ASM thickness occurs through expansion of matrix components rather than contractile elements, as has been observed in patients with fixed airflow limitation.⁷ Volume fractions of ASM and ECM are estimated by use of ultrathin sections (0.5 μm), preventing overlap with tissue viewed in a different plane. Expansion of ECM in preference to ASM would be reflected by an increase and decrease in V_{VECM} and V_{VASM} , respectively. The V_{VASM} and V_{VECM} were however constant over the age range, with only a small deviation in V_{VOTHER} (not significant after post hoc analysis), the parameter most likely to be impacted by histological

artefact (i.e., spaces between tissue). Therefore, the composition of the ASM layer was not affected by age, suggesting that ASM (contractile elements) and ECM increase concomitantly and proportionally with age.

Structural modelling (as opposed to pathological remodelling) of the ASM layer with maturation is expected to alter ASM force production and airway narrowing. Longitudinal data show that in children without asthma, airway responsiveness to contractile stimuli decreases from infancy to age 11 years.¹⁷ Similarly, in other species, airway responsiveness is increased in the immature compared with the mature animal.^{18,19} On the surface, an increase in the thickness of the ASM layer with age is discordant with a decrease in airway responsiveness, although the structure–function relationship is modified by numerous age-dependent factors including subject and airway size,²⁰ contractility (force per cross-sectional area) of the ASM layer,²¹ airway–parenchymal tethering,²² bronchodilatory response to breathing stresses²³ and mechanical loads opposing bronchoconstriction.^{24,25} In particular, while there is a directly proportional relationship between ASM thickness and tension, according to the Law of Laplace, for a given increase in ASM tension, there will be greater collapse pressure in a smaller immature airway compared with a large mature airway.

It is our assertion that understanding the structural mechanism of normal ASM growth provides insight into the pathophysiology of airway remodelling in respiratory disease. Thickness of the ASM layer is increased in asthma,² sudden infant death syndrome (SIDS)²⁶ and chronic obstructive pulmonary disease.²⁷ In asthma, remodelling of the ASM layer is observed in early life³ which implicates maturational time points as the critical window for disruption by disease processes. Appreciating the central role of ASM remodelling in increasing airway narrowing capacity,¹ rapid and disproportionate growth of the ASM layer in the first year of life will likely increase the probability of an asthma diagnosis. This speculation is supported circumstantially by the association between increased airway responsiveness in the first year of life and subsequent asthma development.⁵

Access to rare human airway tissue is difficult and there is a scientific compromise, in particular the unknown effects of various uncontrolled extraneous factors such as cause of death and relative maternal health. Reported causes of death were varied and incomplete, which at the very least would have increased the variability within and between age groups. Infants dying from SIDS have increased ASM compared with non-SIDS infants²⁶ and were therefore not included in this study. There is also a compelling argument that maternal health affects foetal lung growth and increases the susceptibility to airway disease.^{28–30} Adverse foetal lung growth could certainly extend to the ASM layer. For example, maternal hypoxia in mice restricts foetal growth and increases ASM thickness.³¹ The above confounders are acknowledged, albeit there is no reason to suggest that random error affects the trajectory and mechanism of ASM growth, and therefore the conclusions drawn from the present study.

In summary, this study described several phases of ASM development that account for the increase in ASM thickness from late gestation to childhood. Specifically, we observed early ASM hypertrophy and subsequent hyperplasia, and an accompanying expansion of the ECM, such that the overall composition of the ASM layer remained unchanged. Characterizing these structural mechanisms governing normal ASM growth helps advance our understanding on the impact of perinatal influences on airway development and the onset of variable or fixed airflow obstruction in childhood and later life.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Kimberley C. W. Wang: Data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (equal); project administration (lead); resources (lead); software (lead); supervision (lead); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Graham M. Donovan:** Data curation (equal); formal analysis (equal); methodology (equal); software (equal); visualization (equal); writing – review and editing (supporting). **Sejal Saglani:** Data curation (supporting); investigation (supporting); writing – review and editing (supporting). **Thais Mauad:** Data curation (supporting); investigation (supporting); writing – review and editing (supporting). **Alan L. James:** Conceptualization (supporting); data curation (supporting); funding acquisition (supporting); investigation (supporting); methodology (supporting); software (supporting); supervision (supporting); writing – original draft (equal); writing – review and editing (equal). **John G. Elliot:** Conceptualization (supporting); data curation (supporting); formal analysis (supporting); funding acquisition (supporting); methodology (supporting); software (supporting); writing – review and editing (supporting). **Peter B. Noble:** Conceptualization (equal); formal analysis

(equal); funding acquisition (lead); investigation (supporting); methodology (supporting); resources (supporting); software (supporting); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

HUMAN ETHICS APPROVAL DECLARATION

This study was approved by the institutional ethics committees of the participating centres and from the Sir Charles Gairdner Hospital Human Research Ethics Committee (HREC 2015-53).

ORCID

Kimberley C. W. Wang  <https://orcid.org/0000-0002-8604-5909>

Alan L. James  <https://orcid.org/0000-0002-6018-0547>

John G. Elliot  <https://orcid.org/0000-0002-4899-2948>

Peter B. Noble  <https://orcid.org/0000-0001-9028-7751>

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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