Composition of the Airway Smooth Muscle Layer in Airway Disease: Implications for Airway Function and Disease Pathogenesis

By

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Dedicated to Caroline Lesley Johnson

This thesis is presented for the degree of Doctor of Philosophy at The University of Western Australia, School of Medicine and Pharmacology, October 2013
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The publications that have arisen from the work contained in this thesis include one first author, two second author and one third author publication. My specific contributions to each of these papers are detailed below. My conference participation including poster presentations and talks are detailed in Appendix 1.


   For this publication, I performed most of the work including the study design, tissue preparation, histological and stereological measurements, and data analysis and manuscript preparation. Mr John Elliot provided advice on stereological measurements and technical considerations. Professor Alan James assisted with data analysis and manuscript preparation. The data from this paper is presented in chapter 3.

2. **Noble PB, Jones RL, Cairncross A, Elliot JG, Mitchell HW, James AL, McFawn PK**, *Airway narrowing and bronchodilation to deep inspiration in bronchial segments from subjects with and without reported asthma*. J Appl Physiol. 2013 May 15;114(10):1460-71

   This study was predominantly an *in vitro* experiment of isolated human airway segments on well characterised subjects. Although the major outcomes for this paper were the *in vitro* results, pre-operative work-up was performed on all recruited subjects to allow characterisation of the tissue specimens. My contribution to this paper began with the *in vivo* subject work-up. This involved selecting suitable subjects for inclusion in the study (those undergoing lung resection surgery), contacting the patients and arranging a time to meet to get consent, performing modified lung function tests, allergy skin prick tests and administration of respiratory questionnaires prior to surgery, liaising with surgeons and theatre staff, collecting the tissue after surgery and liaising with pathology staff in order to obtain an undamaged airway segment. I was also responsible for entering the patient information into a database and de-identifying and securely storing the patient information that was obtained.
Following *in vitro* experiments performed by Dr. Peter Noble, Dr. Peter McFawn and Mrs Alvenia Cairncross, the tissues were fixed to examine airway structure. Myself and Alvenia Cairncross (under my supervision) performed measurements of airway dimensions prior to and after tissue processing to determine the degree of tissue shrinkage. I embedded the processed tissue into wax blocks and performed all cutting and staining of the airway segments. I also photographed airway segments for inclusion in the paper and performed histological measurements of the airway segments including airway size and the area of airway wall components. I also reviewed drafts of the manuscript. The data from this paper is presented in chapter 5.


This was also an *in vitro* examination of isolated human airway segments in subjects with normal lung function. Pre-operative work was performed on all recruited subjects as stated above to allow characterisation of the tissue specimens. My contribution to this paper again involved all *in vivo* subject workup as detailed above. The *in vitro* experiments were performed by Dr. Peter B Noble, Dr. Peter McFawn, Mr Elanovan Needi and Mrs Alvenia Cairncross. The data from this paper is presented in chapters 5 and 8.


This paper represents a collaborative research study using archival post-mortem tissue collected in studies investigating histological changes in the airways and lungs in patients with asthma. For the production of this paper my contributions were cutting and staining the airway segments (n = 155 subjects at least 4 airways per subject), performing airway measurements on both 0.5μm and 30μm sections and assisting with preparation of the manuscript. Mr John Elliot was also involved with the measurement of the airway segments. The data from this paper is presented in chapter 4.
THESIS SUMMARY

Background
The area of the airway smooth muscle (ASM) layer observed on airway cross-sections is increased and contributes most to the increased airway wall area in asthma. An increase in the volume of smooth muscle within that layer is also likely to be the most important contributor to the increased airway responsiveness in asthma. The area of the ASM layer is increased in a number of other respiratory conditions such as chronic obstructive pulmonary disease (COPD), broncho-pulmonary dysplasia and cystic fibrosis. These conditions may be associated with fixed or variable airflow obstruction and respiratory symptoms (e.g. wheeze and cough), however their histories differ in terms of age of onset, causes, symptoms and response to therapy. The increased area of the layer of ASM in asthma and COPD may be due to increased average smooth muscle cell volume (hypertrophy), increased cell number (hyperplasia), increased volume of other elements such as the extracellular matrix (ECM), or a combination of these changes. Since ASM cell hypertrophy or hyperplasia or a change in the ECM may result from different pathological processes, estimation of the size and number of ASM cells and the volume fraction of ECM in intact airways will give insight into possible disease mechanisms and potential new therapies.

Aims
This thesis examines remodelling of the ASM layer in asthma and COPD. The primary aim is to determine the relative contributions of ASM cell size and number, and ECM to the composition of the ASM layer. The secondary aim is to determine the impact of these changes on airway function (airway narrowing and compliance).

Scope
This thesis examined post-mortem and post-operative airway tissue from subjects with asthma of varying severity or with irreversible airflow limitation (“COPD”) defined by post-bronchodilator forced expired volume in one second (FEV<sub>1</sub>). The volume fractions of ASM, ECM and the number and size of ASM cells within the layer of ASM were estimated in thin and thick sections of whole airways using stereological methods. Factors affecting the variability of these estimates were also assessed. In vitro analysis of airway function was performed using isolated airway segments obtained from freshly resected postoperative tissue. Airway narrowing and airway compliance under
static and dynamic conditions were examined and related to airway structure and subject characteristics.

**Results**

Airway size was the most significant source of variation in stereological estimates once adequate sampling had been achieved (i.e. number fields and section thickness). Hyperplasia and hypertrophy of ASM cells and increased ECM all contributed to the increased area of the ASM layer in asthma, and were related to severity but not duration of asthma. In contrast, increased volume fraction of ECM in the ASM layer, but not changes in ASM cells (number or size) was observed in subjects with reduced FEV\(_1\) (COPD). *In vitro*, increased thickness of the ASM layer was positively related to maximal airway narrowing in subjects with asthma, and the volume fraction of ECM was positively related to cigarette smoking and negatively related to airway compliance in subjects with COPD.

**Conclusion**

Asthma and COPD are often viewed as similar diseases as they are both associated with increased airway narrowing, “airway hyperresponsiveness” and airway inflammation and remodelling. Asthma is a disease where there is excessive smooth muscle in the airway wall due to hypertrophy and hyperplasia of ASM cells. This increased muscle layer can shorten excessively but responds well (dilates) to bronchodilators. The airways in COPD have no change in ASM size or number but an increased volume fraction of ECM. These airways also narrow excessively but respond poorly to direct smooth muscle stimulation therapy (bronchodilators). This increase, or change in the type of ECM may stiffen the airways and prevent physical movement of the ASM resulting in fixed airway narrowing. This thesis has demonstrated that the remodelling of the ASM layer is due to different mechanisms in asthma and COPD. With knowledge of the mechanisms by which ASM layer remodelling occurs, potential new targets for the treatment and management of these airway diseases may be revealed.
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1.1 Introduction

Chronic airway disease is a significant source of mortality and morbidity worldwide, accounting for approximately 6.7 million deaths in 2008 (WHO 2008). Airway disease may include but is not limited to asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, bronchitis, broncho-pulmonary dysplasia and cystic fibrosis. These conditions may be associated with fixed or variable airflow obstruction, respiratory symptoms (e.g. wheeze and cough) and the need for long term therapies for adequate disease control. Asthma (variable airflow obstruction and symptoms) and COPD (fixed airflow obstruction and persistent symptoms) are the most common airway diseases and are both characterised pathologically by inflammation and remodelling of the conducting airways (Jeffery 1991). A major component of remodelling is the increased thickness of the airway smooth muscle (ASM) layer compared with normal healthy subjects (James 1997). The increase in the thickness of the ASM layer may be due to an increase in smooth muscle cell volume (hypertrophy), an increase in smooth muscle cell number (hyperplasia), an increase in the extracellular matrix (ECM) or a combination of these processes. Other components of the ASM layer, such as inflammatory cells or blood vessels, could also contribute to the increased thickness. This thesis will examine remodelling of the ASM layer in asthma and COPD to determine the relative contributions of ASM cell size and number and ECM to the composition of the ASM layer and examine, and discuss the implications of these changes in relation to airway function and disease pathogenesis.

1.2 Prevalence, Morbidity and Mortality of Asthma and COPD

Approximately 235 million people worldwide are currently estimated to be suffering from asthma, and it is the most common chronic disease amongst children (WHO 2011). The global prevalence of asthma ranges from 1-18% depending on the country and definition of asthma and it is attributed to 250,000 deaths annually (GINA 2012). Asthma also has substantial social and economic consequences worldwide, being a major source of absence from both work and school in many counties (GINA 2012). Asthma was responsible for 411 deaths in Australia in 2009, which represented 1.60 per 100,000 people or 0.29% of all deaths in Australia in that year (ACAM 2011). The
prevalence of asthma in Australia is high, and per capita is one of the highest in the world (ACAM 2008; To, Stanojevic et al. 2012) (Figure 1.1). In health studies conducted from 2002-2007, 20.2% of adults reported ever having been diagnosed with asthma with 10.2% reporting current asthma (ACAM 2008). The prevalence of asthma is lower in the United States where, according to the 2007 national survey, only 10.9% of the population reported a prior diagnosis of asthma and 7.2% had current asthma (Pleis and Lucas 2009).

![Worldwide Prevalence of Clinical Asthma](image)

**Figure 1.1: worldwide prevalence of clinical asthma (To, Stanojevic et al. 2012)**

In a worldwide survey in 2004, COPD was found to be the fourth leading cause of death, accounting for 3 million deaths or approximately 5.1% of deaths in that year (WHO 2004; WHO 2012). This exceeds predictions made in 1990 that COPD-related deaths would increase from the 12th leading cause of death to the 6th by 2020 (Lopez and Murray 1998). COPD is a major cause of death in Australia, responsible for 4% of all deaths in recent years (ACAM. 2011). Of all respiratory deaths in Australia, approximately 8% can be attributed to COPD, with 5293 people recorded to have died from COPD in 2009 (AIHW 2012). More concerning is that the symptoms of COPD often don’t appear until the pathology is well-advanced and the prevalence is often under-recognised and under-diagnosed. With respect to mortality, COPD is not often listed as the primary cause of death and so COPD rates are also often under-estimated (ACAM. 2011).
The high mortality and morbidity attributed to asthma and COPD and the resulting cost to the health care system necessitates better treatment and prevention options. Currently all therapies for asthma and COPD (with the exception of strategies aimed at reducing exposure to cigarette smoke and burning fossil fuels) are aimed at reducing symptoms but not treating the underlying cause. It seems likely that respiratory symptoms in patients with asthma or COPD are related to remodelling of the conducting airways and thus a better understanding of airway remodelling could help in the development of new and effective therapies or prevention strategies.

1.3 Structure and Function of the Airways

The function of the respiratory system is to transport air from the atmosphere to the alveoli in the lung periphery, where gas exchange occurs with the pulmonary circulation. The upper respiratory passages include the nose and mouth, pharynx, nasopharynx, oropharynx, larynx and trachea. The human respiratory tract consists of approximately 20 orders of branching from the trachea to the alveoli (Figure 1.2). The trachea branches into the left and right primary bronchi (conducting airways), which direct airflow into the left and right lung. The right lung consists of a right upper, middle and lower lobe while the left lung comprises of an upper and lower lobe. The lobes are further divided into segments and then into lobules, the smallest subdivision visible to the naked eye, with the lobules separated by connective tissue (Crystal 1991). The primary bronchi branch into lobular bronchi which supply air to the separate lobes of the lungs before dividing into segmental bronchi which are conduits for their respective segment. Segmental bronchi divide into progressively smaller bronchioles, eventually leading to the smallest conducting airways, the terminal bronchioles (Weibel 1963). Airways that branch at the level of the terminal bronchioles are referred to as respiratory bronchioles, which supply alveolar ducts but are also involved in gas exchange. Gas exchange in the terminal bronchioles is facilitated by ‘outpocketings’ (alveoli) in the discontinuous bronchiole wall. The airway tree’s final transition is to the alveolar sacs which in turn open into the alveoli (Young 2000). The walls of the alveoli contain a dense network of tiny blood vessels. Gas exchange is facilitated by thin alveoli walls, spherical in structure and many in number, providing an estimated surface area of between 78m$^2$ and 82m$^2$ for the exchange of oxygen into the blood stream and the removal of carbon dioxide (Crystal 1991).
The anatomical structures of the airways are designed to ensure a patent airway lumen and thereby prevent obstruction to airflow. Larger conducting airways of the lungs are held open by supporting cartilage while the smaller non-cartilaginous airways are supported by the elastic recoil of the lung tissue that surrounds them and possibly by the tone of the smooth muscle within the airway wall. An airways basic structure can be more clearly defined in a cartilaginous airway, where the borders between the different wall layers are more apparent. The general structure of the airways consists of two main layers, the mucosa and the submucosa (Figure 1.3). The mucosa consists of the epithelium, basement membrane and lamina propria. Connective tissue such as collagen and elastic fibers and blood vessels make up the lamina propria. The submucosa contains the smooth muscle layer, cartilage and adventitia. Finally, the adventitia consists of a thin connective tissue surface external to the cartilage layer that connects the airway to the surrounding lung parenchyma. Mucous glands are also present in the airways on both sides of the smooth muscle layer. These layers are not always easy to define in a human airway as the relative thickness of the various components change as the airway size decreases (Bai, Eidelman et al. 1994). In airway diseases such as asthma and COPD, persistent long-term inflammation may contribute to airway remodelling which causes permanent alterations to normal airway structure (James and Wenzel 2007).
1.4 Assessing Lung Function

Structural and mechanical changes to the airway wall and the lung parenchyma in disease result in abnormal lung function that manifests through changes in lung volumes, alveolar gas transfer, airway resistance and expiratory flow. These changes can be assessed using a number of lung function tests. The most standardised and most convenient method of assessing lung function is spirometry.

1.4.1 Spirometry

Spirometry measures the volume of air inhaled or exhaled at the mouth over time (Miller, Hankinson et al. 2005). Standard derived values are the maximal volume of air forcibly expired from total lung capacity - the forced vital capacity (FVC), and the volume of air exhaled in the first second of a forced expiration (FEV₁). The expiratory manoeuvres are performed in a sitting position immediately following a maximal inspiration. In healthy subjects approximately 80% of the FVC can be expelled in six seconds or less (Barreiro and Perillo 2004). Forced expiratory volumes are dependent upon age (lung function decreases throughout life), gender, height and race. Measured volumes may be compared with predicted volumes derived from the relevant population to determine whether a subject’s lung function falls within the normal range or whether airflow obstruction is present. In healthy subjects, the measured FEV₁ falls between 80% and 120% of the predicted value (Barreiro and Perillo 2004).
Spirometric measurements are typically displayed as a flow-volume trace, however can also be expressed as volume over time (Figure 1.4A). Measurement of FEV$_1$ and FVC are better illustrated on the volume-time plot (Figure 1.4B). The computed FEV$_1$/FVC ratio is used to identify patients with airflow obstruction where the FEV$_1$ relative to the FVC is reduced. The flow-volume curve provides a visual representation of whether obstruction is present, whereby a concave appearance indicates flow restriction (Figures 1.5). Airflow obstruction is likely to be present in subjects with a FEV$_1$ (% predicted) of less than 80% with more severe obstruction having a progressively lower FEV$_1$ (Pellegrino, Viegi et al. 2005). Airflow obstruction can also be determined by comparison with a predicted lower limit of normal. The lower limit of normal is defined as the result of the mean predicted value (FEV$_1$% predicted) minus 1.64-times the standard error from the population on which the reference lung function values are based (i.e. the lower 5$^{th}$ percentile of the reference population) (McCarthy 2012). If a subject’s FEV$_1$% predicted falls below the normal population range they are classed as having airflow obstruction. Although these normalisation approaches provide slightly different results, both are acceptable techniques in determining airflow obstruction provided the approach is clearly defined (Güder 2012).

Figure 1.4: Spirometry can be used to determine the relationship between flow and volume (A), and volume and time (B) over the course of an uninterrupted breath. Traces indicate FEV$_1$, peak expiratory flow (PEF) and vital capacity (diagram from Barreiro and Perillo 2004).
Figure 1.5: Examples of flow volume loop traces from; (A) a normal subject, (B) a normal subject with an end expiratory curve evident of aging, (C) a subject with asthma or COPD displaying moderate airflow limitation in, (D) a subject with COPD displaying severe airflow limitation (images from Miller, Crapo et al. 2005).

Spirometry is often performed before and after the administration of a bronchodilator, such as salbutamol. A bronchodilator response (manifested as an increase in FEV₁ and FVC) provides a way of distinguishing fixed opposed to reversible airflow obstruction. Relaxation of the ASM to bronchodilator stimuli increases airway patency and facilitates greater airflow. A positive response to bronchodilator is determined from
both the percentage increase in FEV$_1$ and the absolute change in millilitres. The precise
definition of a positive response to bronchodilator differs slightly between societies. In
Australia, reversibility of FEV$_1$ is defined as an improvement of FEV$_1$ of $>$12% and
200ml and is typically present in patients with asthma (Pellegrino, Viegi et al. 2005). In
contrast, a lack of reversibility back to the normal range (i.e. persisting airflow
obstruction) is the hallmark of COPD. However there is at times overlap between these
conditions with subjects with asthma exhibiting a fixed airflow obstruction and subjects
with COPD exhibiting some reversibility.

1.4.2 Static Lung Volumes
The limitation of spirometry is that it only measures the volume of air exhaled through
the mouth during a forced expiratory manoeuvre, and provides no information on
absolute lung volume before or after the respiratory manoeuvre. The capacity to
measure ‘static’ lung volumes is also important in determining the severity, time course
and response to treatment of lung disease (Wanger, Clausen et al. 2005). It may also
provide information on the underlying cause, particularly when combined with
spirometry and gas transfer. Measurements of absolute static lung volumes, including
residual volume, functional residual capacity, and total lung capacity (Figure 1.6) can be
obtained by body plethysmography, gas dilution or nitrogen washout techniques.
Functional residual capacity refers to the volume of gas present in the lung after passive
expiration during tidal breathing at rest; residual volume is the volume of gas remaining
in the lung after a maximal expiratory manoeuvre; and total lung capacity refers to the
volume of gas in the lungs after a maximal inspiration. Importantly, the volume of air
remaining in the lung after a maximal expiration is influenced by whether the
manoeuvre is forced or not, due to dynamic airway collapse (i.e. dynamic airway
collapse occurs during forced expiration and may increase residual volume). Other lung
volume measurements that can be determined include inspiratory capacity; the volume
change to full inspiration following a tidal breath, and slow vital capacity (opposed to
forced vital capacity); the change in volume between a full inspiration and a slow full
(non-forced) expiration (Miller, Crapo et al. 2005). Determination of functional residual
capacity (FRC) is a key component in measuring lung volumes as all other lung
volumes can be readily calculated once FRC is known.
Plethysmography measures lung volume based on Boyle’s Law, which states that when a constant mass of gas is compressed, the gas volume decreases and gas pressure increases so that the product of volume and pressure remains constant (Coates, Peslin et al. 1997; Wanger, Clausen et al. 2005). Subjects are required to sit in an airtight box in which pressure and volume changes can be measured during breathing manoeuvres. As the subject inhales, the volume of gas in the box decreases and the pressure increases. Lung volume (FRC) is calculated from the slope of the relationship between change in lung volume displaced in the box and the mouth pressure before and after the breathing manoeuvre.

The nitrogen wash out technique involves measuring the amount of nitrogen exhaled from the lungs (‘washed out’), while the patient inhales 100% oxygen. A patient’s lung volume is calculated by knowing the initial nitrogen concentration present in the alveoli and the amount of nitrogen ‘washed out’ of the lungs. This technique can be performed by single, or multiple breaths (Wanger, Clausen et al. 2005), with the latter considered more accurate. Finally, the dilution technique can also be used to measure FRC, and is based on equilibration of a known volume of an inert gas (such as helium) with the volume of gas in the lung (Wanger, Clausen et al. 2005). In this technique, the patient breaths from a known volume and concentration of helium. Lung volume can be calculated by measuring the concentration of helium when it reaches equilibrium with the air within the lung.
1.4.3 Gas Transfer

The ability of the lung to exchange gas with the atmosphere may be assessed using carbon monoxide (CO). The carbon monoxide diffusing capacity measures the capacity of the lung to transfer CO from the alveoli into the pulmonary capillary blood (Gardner, Crapo et al. 1988). This measurement relies on the physical properties of CO and an inert gas mixture (usually helium or methane). Gas transfer assessment requires subjects to take a deep breath of a CO and inert gas mixture, hold the breath for approximately ten seconds and breathe out again (Behr 2008). Following inspiration the CO rapidly diffuses from the alveolar into the blood and binds to haemoglobin, while the inert gas remains in the alveolar space. The concentrations of CO and inert gas in the inspired and exhaled gas mixture can be used to calculate carbon monoxide diffusing capacity and determine the rate at which CO is taken up by the lung. Measuring the CO uptake is useful in assessing airway and vascular disease where abnormalities may impair the capacity of the alveoli to transport gas.

1.5 Asthma

Asthma is a chronic airway disease characterised by excessive and reversible airway narrowing, airway inflammation, remodelling and increased airway responsiveness to bronchoconstricting stimuli and, in many cases, reduced lung function (Begueret, Berger et al. 2007; Tliba and Panettieri 2008; Rubin 2001; Robertson 2002). Unlike other chronic airway diseases, the symptoms of asthma are reversible either spontaneously or with treatment (Bateman, Hurd et al. 2008). The diagnosis of asthma is based mainly on a history of persistent or intermittent clinical symptoms which may vary over time or with treatment. Predominant symptoms include wheeze, chest tightness, shortness of breath, cough and mucus production (James and Wenzel 2007). Symptoms of asthma can have a variety of different triggers. The most common triggers include cigarette smoke, exercise, cold air, viral respiratory infections and inhaled allergens (e.g. pollens, moulds, animal hair and dust mite). Specific but unusual triggers that are important to recognise include occupational exposures (aldehydes, laboratory animals, western red cedar, isocyanates), sulphur dioxides and aspirin (and other non-steroidal anti-inflammatory medications). Due to the wide range of triggers and diversity of symptoms associated with asthma, diagnosis also require assessment of the reversibility of airway obstruction or assessment of airway responsiveness.
Asthma severity is based on the frequency and severity of symptoms and may range from intermittent to severe/persistent (Table 1.1). For classification of asthma severity, symptoms within the last 12 months are usually assessed. For research studies, asthma severity questionnaires are available that consider symptom frequency, medication use and asthma-related exacerbations and hospitalisations. During an exacerbation of asthma, the airways are infiltrated with inflammatory cells including eosinophils and/or neutrophils and lymphocytes associated with vascular extravasation. Plugs of mucus and inflammatory and epithelial cells occlude the lumen and the ASM shortens, producing airflow obstruction. As discussed, inhaled bronchodilator agents, most often β-2 agonists, act to relax the ASM and alleviate symptoms. Other medications such as inhaled corticosteroids (“preventers”), long-acting β-2 agonists (“controllers”) and monoclonal anti-IgE therapy (“disease modifiers”) can also be used, depending on the severity of the asthma (Hoshino, Toda et al. 2009; Bateman, Hurd et al. 2008).

Table 1.1: Classification of asthma severity using reported symptoms and results from lung function testing (adapted from O’Byrne, Bateman et al. 2007).

<table>
<thead>
<tr>
<th>Asthma Classification</th>
<th>Day Symptoms</th>
<th>Night Symptoms</th>
<th>FEV₁ % predicted</th>
<th>PEF variability</th>
<th>Attacks and activity levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>&lt;1 per week</td>
<td>&lt;2 per month</td>
<td>&gt;80%</td>
<td>&lt;20%</td>
<td>Asymptomatic, normal airflow attacks</td>
</tr>
<tr>
<td>Mild Persistent</td>
<td>&gt;1 per week</td>
<td>&gt;2 per month</td>
<td>&gt;80%</td>
<td>20 - 30%</td>
<td>Attacks may affect activity</td>
</tr>
<tr>
<td></td>
<td>&lt;1 per day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate Persistent</td>
<td>Daily</td>
<td>&gt;1 per week</td>
<td>60-80%</td>
<td>&gt;30%</td>
<td>Attacks do affect activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe Persistent</td>
<td>Continuous</td>
<td>Frequent</td>
<td>&lt;60%</td>
<td>&gt;30%</td>
<td>Limited physical activity</td>
</tr>
</tbody>
</table>

PEF - Peak Expiratory Flow; FEV₁ - Forced Expiratory Volume in the first second.

It is important to note that whilst asthma treatments are effective in controlling symptoms, they do not reverse the underlying structural abnormalities (i.e. airway remodelling). While there is evidence that inhaled corticosteroids reduce reticular basement membrane thickening (Hoshino, Nakamura et al. 1998), other features of
remodelling may be less responsive to current treatments, although there are few data available. Additionally, although asthma is often known as a reversible disease, persistent inflammation and remodelling in asthmatic subjects is associated with irreversible airflow obstruction which may lead to the subsequent diagnosis of COPD.

1.5.1 Lung Function in Asthma

There is good evidence to suggest that reductions in lung function in individuals with asthma occur early in the clinical course of the disease. Data from cross-sectional studies show that lung function is reduced in children with asthma (Sears, Greene et al. 2003; CAMP 2000; Oswald, Phelan et al. 1994) and is related to the severity of asthma and frequency of symptoms. Abnormal lung function appears to be present at birth in those who later develop persistent wheezing and asthma (Guilbert, Singh et al. 2011; Palmer, Rye et al. 2001). Lung function abnormalities detected in early life are related to asthma severity and often persist into adulthood, generally remaining stable over time (Robertson 2002; Sears, Greene et al. 2003).

Longitudinal studies have examined the effect of asthma on the decline in FEV₁ which occurs in all individuals from the age of about 20 years. Some studies have suggested that asthma is associated with an increased rate of decline (Lange, Parner et al. 1998; James, Palmer et al. 2005) whereas others have not (Robertson 2002; Sears, Greene et al. 2003). Confounding factors such as cigarette smoking, occupational exposure to airborne stimuli and additional lung disease make disease progression difficult to assess. Studies on asthmatic subjects who smoke show a faster decline in lung function compared with non-smoking individuals and smoking non-asthmatic subjects (Figure 1.7) (Grol, Gerritsen et al. 1999; James, Palmer et al. 2005). Treatment with inhaled corticosteroids improves lung function in asthma (Parameswaran, O'Byrne et al. 2003; Donohue and Ohar 2004) but does not necessarily alter the rate of decline in FEV₁ (CAMP 2000), although improvement has been observed in some studies (Lange, Scharling et al. 2006; Dijkstra, Vonk et al. 2006).
Figure 1.7: Age related decline in FEV$_1$ of male asthmatic non-smokers (solid line), non-asthmatic non-smokers (dotted line), asthmatic smokers (dotted and dashed line) and non-asthmatic smokers (dashed line) (James, Palmer et al. 2005).

While there are conflicting reports as to whether there is an increased rate of decline in lung function in patients with asthma, any accelerated decline is relatively small in patients with mild to moderate symptom severity (Sherrill 2003). The vast majority of patients with asthma can attain excellent control of symptoms and maintain near-normal lung function with the use of effective and safe medication.

1.5.2 Radiology in Asthma

The altered appearance of chest radiographs in asthma has been noted for many years (Hodson, Simon et al. 1974), but it is only with the development of high resolution computer tomography (HRCT) that these changes have been fully appreciated. Airway remodelling, a characteristic feature of asthma, is visible on CT scan as bronchial wall thickening and narrowing of the bronchial lumens (Silva, Colby et al. 2004; Lynch, Newell et al. 1993; Grenier, Mourey-Gerosa et al. 1996; Park, Muller et al. 1997). Other abnormalities that have been observed include areas of decreased attenuation and vascularity, hyperinflation, and air trapping as seen on expiratory CT scans (Lynch, Newell et al. 1993). The related magnitude of bronchial wall thickening identified by HRCT varies from 44 – 92% due to the highly subjective nature of the CT examination. Approximately 30 – 40% of adults with uncomplicated asthma also show one or more
dilated bronchi (Silva, Colby et al. 2004). Approximately half of all patients with asthma have areas of air trapping on a CT scan, making this the most common abnormality observed. Areas of decreased attenuation and vascularity can also be viewed on end-expiration CT scans, however these features are only seen in approximately 20% of patients with asthma (Park, Muller et al. 1997).

1.5.3 Asthma Pathology
Histopathologic studies have shown that changes are present in both the central and peripheral airways of subjects with asthma. These changes involve both cellular infiltrates and structural changes to the airway wall. Asthma is pathologically characterised by intraluminal mucous and cellular debris, goblet cell hyperplasia, submucosal inflammation of neutrophils, eosinophils and lymphocytes and airway wall remodelling (Fireman 2003; Rubin 2001). The predominant features of airway remodelling in asthma are increased thickness of the ASM layer (Figure 1.8) and general thickening of the inner and outer wall compartments, possibly due to inflammation (James 1997). Mucous gland hypertrophy and increased vascularisation of the airways also contributes to the increased airway wall thickness (Saetta and Turato 2001). Excessive secretions of mucous caused by both mucous gland and goblet cell hypertrophy is another feature of asthma that contributes to obstruction of the airway lumen. The increased thickness of the airway wall in asthma is likely to alter airway mechanics including airway narrowing to bronchoconstrictor stimuli (Section 1.7).

The inflammatory response of the airways in asthma most often involves “Th2” cytokines (interlukins IL-5 and IL-13) which promote eosinophilic proliferation and cellular migration to the lung (Umetsu and Dekruyff 2010). Although the inflammatory response occurs throughout the entire airway tree, the distribution of cellular infiltrate may differ between the small and large airways (Saetta and Turato 2001). Eosinophilic inflammation is located primarily in the inner airway wall of large airways (between the ASM layer and the basement membrane), while in the small airways, inflammation occurs outside the ASM. Neutrophilic infiltration has also been demonstrated in subjects with asthma (Fahy 2009). The presence of predominantly eosinophilic or neutrophilic inflammation may represent distinctly different asthma phenotypes, despite the similar clinic features (Wenzel 2006; Pavord, Brightling et al. 1999; Green, Brightling et al. 2002).
Figure 1.8: Normal (A) and asthmatic (B) bronchi. In the asthmatic bronchus the ASM layer is thickened, the airway is inflamed and swollen, and mucous plugs obstruct the airway lumen.

1.6 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a preventable disease (in many instances) characterised by airflow limitation which is not fully reversible, airway inflammation, and remodelling often with associated symptoms such as wheeze, chronic cough, difficulty breathing (dyspnoea) and sputum production (Mannino 2002; Mannino 2003; Celli and MacNee 2004). The degree of airflow limitation is related to the severity of disease and is associated with an abnormal inflammatory response to noxious particles or gases (Celli and MacNee 2004). COPD is a condition that tends to become apparent later in life, usually in association with cigarette smoking but can also be caused by asthma, bronchiolitis, broncho-pulmonary dysplasia, air pollution and other exposures (Gibson and Simpson 2009; Postma and Kerstjens 1998). Of these risk factors, tobacco smoke exposure is by far the most common cause with approximately 20% of smokers in developed countries having measurable airflow obstruction (Buist, McBurnie et al. 2007). Given that lung function also declines with age, the true impact of COPD may be more apparent as the world’s population ages. COPD is already a leading cause of death and a major burden on health care systems throughout the world (Rabe, Hurd et al. 2007) and will continue to be so, especially if current smoking rates persist.
The presence of COPD may be suspected based on clinical symptoms such as progressive and persistent breathlessness on exertion, especially when history of cigarette smoking is reported. Diagnosis of COPD is confirmed by spirometry and is indicated by a low FEV₁ and FEV₁/FVC ratio (Mannino 2002). The presence of airflow limitation that is not fully reversible can be confirmed by a post-bronchodilator FEV₁/FVC of $\leq 0.7$ (Celli and MacNee 2004; Rabe, Hurd et al. 2007). Sub-classification of COPD as mild, moderate, severe and very severe are based on the FEV₁ relative to the predicted value and can help in treatment planning and predicting outcomes of morbidity (Table 1.2) (Decramer 2012). Body mass index and the degree of breathlessness are also known predictors of patient outcomes (Celli and MacNee 2004). The global initiative for obstructive lung disease (GOLD) has developed a global classification system for COPD with the aim of providing guidelines for the diagnosis and treatment for the different grades of COPD for patients around the world (Decramer 2012). However, mild COPD does not usually present with clinical symptoms. Because of this, its prevalence and morbidity tends to be greatly underestimated in studies of the global burden of disease (Mathers 2008).

Table 1.2: COPD disease severity is based on and FEV1 / FVC ratio of <0.7 and FEV₁ % predicted. (Decramer 2012)

<table>
<thead>
<tr>
<th>Severity</th>
<th>FEV₁ % Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>$\geq 80$</td>
</tr>
<tr>
<td>Moderate</td>
<td>50 – 80</td>
</tr>
<tr>
<td>Severe</td>
<td>30 – 50</td>
</tr>
<tr>
<td>Very Severe</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

1.6.1 Lung Function in COPD

The distinguishing feature of COPD for the most part is fixed airway obstruction, showing little to no improvement in FEV₁ after bronchodilator inhalation (Toelle, Xuan et al. 2013). The use of a fixed system of COPD diagnosis, as recommended by GOLD, provides consistency in the diagnosis of COPD worldwide (Rabe, Hurd et al. 2007). These criteria have been used in many clinical trials and are used to form treatment recommendations for different COPD severities. As discussed, COPD can also be defined using the lower limit of normal values for FEV₁/FVC, rather than a fixed cut
off. These lower limit values are based on the normal distribution of lung function in a large population where the bottom 5% of this population is classified as ‘abnormal’. There are some discrepancies in diagnosis between the two methods of classification, making it difficult to discern which method is more correct. Defining COPD using a fixed cut off (i.e. <0.7 FEV$_1$/FVC) will generally result in more older people being diagnosed with COPD, whereas diagnosis based on the lower limit of normal will vary considerably depending on the reference population used and often results in under-diagnosis in the younger population (Güder 2012).

In addition to detection of flow limitation by spirometry, lung hyperinflation and changes in alveolar diffusion capacity are resent in some patients with COPD. Peripheral airway obstruction in COPD can cause gas trapping during expiration, resulting in hyperinflation of the lung (Rodriguez-Roisin, Drakulovic et al. 2009). Hyperinflation can be detected using plethysmography, nitrogen wash out or the dilution approach by a rise in residual volume, FRC and/or total lung capacity (Rabe, Hurd et al. 2007). A reduced diffusion capacity may indicate destruction of alveoli (i.e. ‘emphysema’). Carbon dioxide retention can follow reduced alveolar ventilation due to the high work of breathing often experienced by COPD sufferers. An increasingly common approach to assessing the severity of COPD is exercise testing (Oga, Nishimura et al. 2003), as sufferers will often have a low tolerance for exercise due to breathlessness resulting from the remodelling changes of the airways and emphysema of the lungs.

1.6.2 Radiology in COPD

A number of radiographical abnormalities have been detected in COPD patients using techniques such as chest X-rays, standard CT scans and HRCT. The sensitivity to detect such abnormalities varies depending on the technique used. Chest X-rays can provide an initial imaging tool for the assessment of COPD and is useful in excluding other diagnoses such as pneumonia and pleural effusion. However other changes visible on X-ray, such as lung hyperinflation, loss of pulmonary vessels and increased bronchial wall thickness, are not specific enough for a direct diagnosis of COPD to be made (Pipavath, Schmidt et al. 2009). CT scans taken at either inspiration or expiration allow for qualitative and quantitative assessment of the extent, type and lobular distribution of emphysema, and the presence of air trapping within the lung (Miniati, Filippi et al. 1995). While HRCT is the most sensitive and specific tool currently available to assess
radiological abnormalities in patients with COPD (Takasugi and Godwin 1998), conventional CT is sufficient to detect structural alterations in the airways and the lung and is commonly used in the diagnosis of COPD (Miniati, Filippi et al. 1995; Coxson and Rogers 2005; Pipavath, Schmidt et al. 2009).

The main features of COPD visible on CT scans are areas of emphysema, thickening of the bronchial walls and enlarged blood vessels. The severity of COPD can be quantified by measuring lung density, with areas of emphysema defined as those with Hounsfield Units (HU) between –910 and –950HU. The percentage of emphysema determined in this manner can then be used to classify COPD subjects into different disease severities or phenotypes (Coxson and Rogers 2005). Ground glass opacity is a non-specific finding often noted on CT scans of subjects with COPD (Takasugi and Godwin 1998) and is characterised as areas on increased density within the lung, and often indicates fluid filling of the air spaces (Engeler, Tashjian et al. 1993). In the respiratory bronchioles, ground glass opacity can be due to mucous or inflammatory cell infiltration of the alveoli and alveolar walls (Takasugi and Godwin 1998). Additionally CT scans, as used in asthma, are used to measure the dimensions of the bronchial walls, providing another means to assess the presence and severity of airway disease in COPD (Coxson and Rogers 2005).

1.6.3 COPD Pathology

The pathological changes seen in the lungs of patients with COPD include neutrophilic inflammation, infiltration of CD8 T-lymphocytes, thickening of the airway wall and ASM layer (although not generally to the extent seen in asthma), increased blood vessel density, enlargement of the submucosal mucous glands, hypersecretion of mucus, metaplasia of the epithelial cells, loss of terminal and respiratory bronchioles and enlargement and destruction of the alveoli (emphysema) (Hogg, Chu et al. 2004; Hogg, Macklem et al. 1968) (Figure 1.9). Structural changes to the airway wall are more prominent in the small airways (<2mm in diameter) but are also observed (to a lesser extent) in the large airways (Chung 2005; Jeffery 2001). Genetics may play a role in determining the relative structural alterations at each of these airways sites, which varies between patients (Patel, Coxson et al. 2008; Hersh, Hokanson et al. 2011). These structural changes and the loss of peripheral airways (resulting in emphysema) may result in increased resistance to flow. Emphysema also contributes to reduced expiratory drive due to a loss of elastic recoil of the lung parenchyma and loss of support for the
small airways, thus leading to airway collapse upon expiration. The severity of airway remodelling and parenchymal tissue damage combine to determine the effect on airflow obstruction and the clinical severity of COPD. Emphysema also contributes to reduced alveolar surface area and therefore reduced gas exchange and hyperinflation, shortening the diaphragm with subsequent mechanical disadvantage. All of these changes contribute to breathlessness, which is sometimes present at rest but made worse with subject exertion.

![Figure 1.9: Bronchioles from a control (A) and COPD (B) subject.](image)

Figure 1.9: Bronchioles from a control (A) and COPD (B) subject. In the airway from the COPD subject, alveolar attachments to the airway wall are damaged or absent, the epithelium is thickened and the mucous glands are enlarged.

### 1.7 Airway Narrowing

Airway narrowing can occur in response to a wide variety of stimuli including “specific” pharmacological agents such as methacholine and histamine and “non-specific” agents such as cold or dry air. These stimuli exert their effects by acting on the smooth muscle to produce contraction. Excessive airway narrowing is a feature of both asthma and COPD however the underlying mechanisms remain unclear. Airway hyperresponsiveness is characterised by both an increase in airway sensitivity and a greater maximal airway narrowing response (Woolcock, Salome et al. 1984; Sterk, Daniel et al. 1985). It is still unclear if these phenomena are due to changes in the structure of the ASM, changes to the airway wall or in changes to the surrounding lung parenchyma (King, Pare et al. 1999).
As discussed (Section 1.3) the airway wall can be broadly divided into two compartments, the mucosa and the submucosa. The mucosa comprises the area between the airway lumen and the smooth muscle layer and the submucosa encompasses the airway smooth muscle layer, and the area between the outer boundary of the smooth muscle layer and the lung parenchyma. Increased thickness of either of these components, or thickening of the ASM layer, will affect the degree of airway narrowing that occurs in response to a stimulus. Mathematical models and morphometric analyses have shown that changes within each of these compartments can contribute to increased airway narrowing and airway hyperresponsiveness (Lambert, Wiggs et al. 1993; Moreno, Hogg et al. 1986; Oliver, Fabry et al. 2007; Wiggs, Bosken et al. 1992). The relative functional implications of these morphological changes in asthma and COPD are yet to be fully established.

1.7.1 ASM Contraction

The ASM cell consists of overlapping contractile elements, actin (thin filament), and myosin (thick filaments) anchored to plaque like structures known as dense bodies (Becker 2003). These dense bodies are interconnected by intermediate filaments and are arranged longitudinally in the cell. Contraction of the ASM occurs via a process known as cross-bridge cycling which involves sliding of the actin and myosin filaments and the production of force and shortening of the muscle fibres (Figure 1.10). This process is activated through an increase in intracellular calcium which promotes a cascade of events, including the activation of myosin light chain kinase (MLCK). Activated MLCK then phosphorlylates myosin II, enabling it to interact with actin filaments and undergo cross-bridge cycling. During cross-bridge cycling, the myosin heads tilt and slide along the actin filament. The myosin head then detach from the actin filament and binds to another site further down the actin filament, facilitating shortening (Figure 1.11). As intracellular calcium levels fall, MLCK is inactivated and the phosphate is removed from myosin II. When myosin II is in it’s dephosphorylated state, myosin can no longer bind to actin and the muscle cell relaxes (Becker 2003). There is evidence to suggest that the rate of ASM contraction may vary depending on the length of the ASM cell, contracting faster when there are more contractile units (containing myosin filaments) engaged in series (Seow and Pare 2007) (see below). In airway smooth muscle, contractile filaments are thought to be able to rearrange themselves by increasing the overlap between the thick and thin filaments in order to maximise force production for a given length (see ASM length adaptation) (Seow and Pare 2007).
1.7.2 ASM Length Adaptation

Striated muscle has a well-characterised and relatively fixed relationship between resting length and the force that can be developed following maximal stimulation, such that there is an optimal length (related to overlap of actin and myosin filaments) at which maximal force can be produced by the muscle. Non-striated smooth muscle also exhibits a length-force relationship, but due to the process of ‘length adaptation’ the relationship is not a constant and any length can theoretically produce maximum force (Wang, Pare et al. 2000). ‘Length adaptation’ is the ability of smooth muscle to optimise force over an extended length range (Kuo, Herrera et al. 2003; Bosse, Sobieszek et al. 2008; Ali, Chin et al. 2007; Naghshin, Wang et al. 2003). Evidence to date suggests that length adaptation involves reassembly of the contractile elements.

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Figure 1.10: Arrangement of the ASM contractile filaments in a relaxed (A) and contracted (B) state. Thick filaments (myosin) and thin filaments (actin) interact to allow the smooth muscle to shorten, creating tension on the elastic and non-elastic non-contractile tissue elements connected in series with the smooth muscle.

Figure 1.11: Schematic illustrating filament interactions producing ASM contraction. The myosin head is activated through phosphorylation and binds to actin (A). The myosin head tilts and slides along the actin filament (B). The head dephosphorylates, releases the actin filament which binds subsequently at a site further along the myosin filament (C).
within the smooth muscle cells to optimise the overlap of myosin and actin filaments at any given length (Bosse, Sobieszek et al. 2008). This process is believed to be triggered by strain on the cell cytoskeleton sensed by transmembrane proteins referred to as integrins. Complete adaptation can occur within 30 minutes during repetitive smooth muscle contraction, however the process of length adaptation likely begins within seconds of an imposed length change (Wang, Pare et al. 2000). Length adaptation can also occur in relaxed muscle when held at a fixed length, however this process occurs over a period of hours (Wang, Pare et al. 2001) or days (Naghshin, Wang et al. 2003). The result of the smooth muscle adaptation is a shift in the length force relationship of the cell, allowing it to generate maximal force at the new length (Figure 1.12). This ability of the ASM can best be explained through the use of a model where the contractile units of the cell are arranged in parallel (Bosse, Sobieszek et al. 2008). Following contractile stimulation, these contractile units partially disassemble before realigning at a new length by adding or subtracting the number of actin and myosin filaments involved in order to optimise force production (Figure 1.13). Long term adaptation is proposed as a mechanism that could lead to excessive airway narrowing by causing increased muscle stiffness and a greater force-generating capacity at short lengths (Naghshin, Wang et al. 1985).

![Figure 1.12: Force-length adaptation of the ASM. If the ASM is acutely shortened beyond its maximal force producing length (A), the force produced is decreased (B). When the muscle is held at the new shortened length, muscle adapts to optimise force production at this new length (C) (adapted from Wang, Pare et al. 2001).](image-url)
Figure 1.13: Proposed arrangement of the contractile units within the ASM cell in a lengthened and shortened state. The actin and myosin units become reorganised in order to achieve maximum force production at any given length (adapted from Kuo, Herrera et al. 2003).

1.7.3 Mechanics of Airway Narrowing

As discussed in Section 1.5, airway narrowing in response to a bronchoconstricting stimulus is excessive in patients with asthma as well as showing increased sensitivity and reactivity. These phenomena are collectively known as airway hyperresponsiveness. In contrast, in patients with COPD the airways appear to be in an excessively narrowed and fixed state. It is not clear to what extent airway hyperresponsiveness, as observed in asthma, contributes to airflow limitation in COPD. In addition, changes in the elastic recoil of the lung contribute to excessive airway narrowing in COPD, especially during expiration.

Airway narrowing occurs by a series of pharmacological and mechanical events (Figure 1.14) (Moreno, Hogg et al. 1986). Contractile agonists activate the muscle by binding to receptors on the cell. Access to the ASM is via the mural blood circulation or by diffusion through the epithelial barrier present at the luminal surface. The magnitude of smooth muscle force development after stimulation is determined by the affinity of the agonist to the receptor, the length tension characteristics of the smooth muscle and the volume of smooth muscle that is present in the airway wall. The degree of shortening
that occurs for a given amount of force generation is determined by the mechanical
loads that oppose ASM shortening. Narrowing of the airway luminal space is a direct
consequence of ASM shortening and is dependent on the thickness of the airway wall,
and the presence of mucus in the airway lumen. Interactions of airway narrowing with
the series and parallel resistances in the bronchial tree will reduce airflow detected by
lung function testing (Moreno, Hogg et al. 1986).

![Diagram of ASM Activation and Airway Resistance](image)

Figure 1.14: Factors influencing airway narrowing and airway resistance (adapted from
Moreno, Hogg et al. 1986).

Airway narrowing in response to an inhaled contractile stimulus is often expressed in
the form of a dose-response curve, from which a number of parameters can be derived
to describe its shape and position. These include the sensitivity which is the position of
the curve in relation to the dose axis, the reactivity which is the slope of the linear part
of the curve (the amount of response for a given dose) and the maximal response or
plateau response (Moreno, Hogg et al. 1986) (Figure 1.15). In the laboratory the
provocative dose causing a 20% fall in FEV₁ (the PD20) is most often used as a single
measure of hyperresponsiveness and is itself determined by the sensitivity, the reactivity
and the maximal response (O'Connor, Sparrow et al. 1987). Since many subjects will
not achieve a fall of 20% or more, many subjects’ data are censored using this approach.
Another measure that is used is the dose-response slope, which is the largest response
divided by the dose at that response (O'Connor, Sparrow et al. 1987). This allows all
subjects’ data to be used as continuous variables. Woolcock and colleagues (Woolcock,
Salome et al. 1984) demonstrated that a plateau was reached in both normal individuals
and those with mild asthma, however the maximal response in asthma was increased.
Subjects with moderate or severe asthma do not show a plateau and FEV\(_1\) falls substantially at low concentrations of methacholine. Similar findings on non-asthmatic and asthmatic subjects have also been reported in other studies (Currie, Fowler et al. 2003; Lipworth 1996; Henriksen, Lingaas-Holmen et al. 2000). In comparison to asthma, a plateau of the dose-response curve is reached in COPD where no further airway narrowing occurs regardless of the dose given (Postma and Kerstjens 1998). The plateau, when expressed as percent change from baseline, is elevated in COPD compared with normal subjects, but not to the extent observed in asthma.

![Figure 1.15: Schematic of a dose response curve for a normal, COPD and asthmatic subject. Severe asthma exhibits greater responsiveness than mild asthma or COPD. Airway responsiveness in COPD is greater than in normal subjects but less than in asthma.](image)

Despite the similarities between asthma and COPD, the mechanisms which cause airway narrowing in these diseases are likely to differ. There are many different mechanisms which can cause airway narrowing, including increased wall area, mucous plugging, excessive ASM shortening and airway collapse due to loss of parenchymal attachments (Figure 1.16). Both asthma and COPD show inflammation of the airway, and in asthma a relationship exists between the degree of airway inflammation and the severity of airway hyperresponsiveness (Gronke, Kanniess et al. 2002). In asthma the inflammation causes airway wall oedema, deposition and remodelling of connective tissue, hypertrophy and hyperplasia of smooth muscles cells and new blood vessel formation (James 2005). These changes result in airway wall thickening, and may also increase airway wall stiffness due to biochemical changes in tissues, which in turn affect
smooth muscle contraction (Niimi, Matsumoto et al. 2003), discussed further below. COPD is characterised by chronic inflammation leading to destruction of the lung parenchyma and decreases in elastic lung recoil (Postma and Kerstjens 1998). The inflammatory cell type and location in COPD may also increase the collapsibility of the airways making them more susceptible to closure upon expiration. The remodelling of airway wall tissues and ASM in asthma and COPD contributes to the mechanical properties of the airway wall and the degree of airway narrowing and airway hyperresponsiveness in these diseases.

![Figure 1.16: Possible airway changes contributing to excessive airway narrowing.](image)

In the review by Moreno et al (Moreno, Hogg et al. 1986) several mechanisms for excessive airway narrowing were highlighted, including increased ASM contractility, decreased mechanical load on the ASM (e.g. decreased tethering of the lung parenchyma), increased ASM mass, increased wall thickness and increased secretions in the airway lumen. Although all of these changes favour increased airway narrowing, mathematical models show that an increase in airway wall thickness had the most profound effect on the degree of airway narrowing (Wiggs, Bosken et al. 1992). Even modest increases in the thickness of the airway wall produced a profound increase in airway narrowing caused by increased smooth muscle shortening, especially in the smaller airways (Postma and Kerstjens 1998). In the model, when the inner airway wall was thickened, airway resistance was modestly increased at baseline (i.e. prior to ASM contraction) (Figure 1.17) (Postma and Kerstjens 1998). However, in the presence of simulated smooth muscle shortening, the increase in wall thickness produced a substantially greater increase in airway resistance. Thickening of the outer layer has a similar effect on airway resistance, although it operates through a different mechanism (Figure 1.18). In normal airways, when ASM contraction occurs, the amount of airway narrowing is limited by the outward recoil of the surrounding lung parenchyma. When the outer airway wall is thickened the elastic recoil pressure on the airway wall is
reduced, allowing for greater smooth muscle shortening and airway narrowing (Kuwano, Bosken et al. 1993).

![Diagram of inner wall thickening](image1.png)

**Figure 1.17:** The effect of inner wall thickening. In this example, the normal airways have an inner wall area of 20%. When 30% ASM shortening occurs, the airway resistance (R) increases. An increase in the wall area to 40% produces a small increase in resistance at baseline. The same degree of ASM shortening (30%) of the thickened airway increases resistance to 80 times baseline (Kuwano, Bosken et al. 1993).

![Diagram of outer wall thickening](image2.png)

**Figure 1.18:** The effect of outer airway wall thickening. In this example the normal airway (above) has a resistance (R) of one unit. When ASM contraction is stimulated, shortening occurs until the parenchyma (represented by the springs) prevents further shortening at a resistance of eight. In the airway below, outer wall thickening reduces the tension in the springs and airways can narrow until shortening is restricted at a greater resistance of 50 units (Kuwano, Bosken et al. 1993).

The amount of smooth muscle within the airway wall has also been found to be an important contributor to airway narrowing, especially in the small airways (Lambert, Wiggs et al. 1993). An increased amount of ASM may increase the total force that can
be generated by the muscle, allowing it to overcome the elastic loads opposing smooth muscle shortening. This may apply even if the force-generating capacity of individual ASM cells is not different in asthma. Several studies have shown no difference in the force produced by ASM from subjects with asthma compared with non-asthmatic subjects (De Jongste, Mons et al. 1987; Bai 1991; Vincenc, Black et al. 1983; King, Pare et al. 1999). ASM cells may undergo changes in their phenotype and function in response to physiological cues (Hirota, Nguyen et al. 2009). These cells have an increased proliferative capacity and a decreased contractile apparatus, consistent with reduced contractile responses in vitro (Gosens, Meurs et al. 2002; Dekkers, Schaafsma et al. 2007). These data suggest that increased proliferation of ASM cells in vivo, if it occurs in asthma, may not necessarily increase the capacity of the ASM layer to produce force despite its increased size. Although there is good evidence in vitro for increased proliferative response of ASM cells from cases of asthma (Johnson, Roth et al. 2001), the evidence for this in vivo is conflicting and unresolved (Ward, Harris et al. 2008; Hassan, Jo et al. 2010).

Recently, the dynamic mechanical environment of the lung has been acknowledged as an important determinant (regulator) of airway narrowing. Historically the majority of studies have examined ASM contraction using static biological models (Gunst 1983; Gunst and Mitzner 1981; Gunst, Stropp et al. 1990; Gunst and Stropp 1988; Okazawa, Bai et al. 1993; Okazawa, Vedal et al. 1995; Macklem 1996; Lambert and Pare 1997). In a static model the length of the muscle and calibre of the airways is viewed as being dependent on the force-length relationship of the muscle, the amount of muscle, static loads of parenchymal tethering, elastic recoil of the lung and the forces required for mucosal folding (King, Pare et al. 1999). Although this model takes into account passive forces, it fails to take into account intracellular loads and the effects of connective tissue on ASM movement (Hirota, Nguyen et al. 2009). There is increasing evidence to suggest that cyclic and periodic stretch are important in attenuating excessive airway narrowing in normal subjects and that the response of the ASM layer to these stretches is abnormal in asthma (King, Pare et al. 1999). Dynamic models of ASM contraction account for loads that the ASM is subject to in vivo, including tidal force oscillations during normal quiet breathing, and periodic deep inspirations (DI) or sighs. These models have allowed for studies of the airway in an environment which more closely mimics the working respiratory system and the stresses applied to the ASM during airway contraction.
Through the use of a dynamic airway model, tidal breathing has been shown to produce ASM relaxation due to cyclical stretching of the ASM (Noble 2012; Raboudi, Miller et al. 1998; West 2012). Application of a DI during tidal breathing is thought to increase the magnitude of relaxation. Fredberg and colleagues (Fredberg, Inouye et al. 1997) predicted that the relaxation of the ASM layer caused by a DI could result in a five-fold increase in ASM length than can be achieved through normal tidal breathing. The ability of DI to relax the ASM layer (bronchodilation) in normal subjects has been observed in several studies. Lengthening of ASM occurs in both relaxed airways and in those with existing tone (Brusasco, Pellegrino et al. 1992), with a deeper inspiration causing a greater bronchodilator response, helping to overcome bronchoconstriction (Salerno, Pellegrino et al. 2005). In subjects with asthma however, the ability of a DI to relax ASM and dilate the airway is reduced (Nadel and Tierney 1961; Fish, Ankin et al. 1981; Kapsali, Permutt et al. 2000; Scichilone, Permutt et al. 2001). It has even been suggested that the inability of the asthmatic airway to bronchodilate with DI may be the main cause of excessive airway narrowing (Fish, Ankin et al. 1981).

Properties of the ASM are likely to influence the response of the airway to different breathing manoeuvres. Fredberg et al. (Fredberg, Inouye et al. 1999) described a conceptual framework that may account for increased airway narrowing due to the failed effects of normal breathing manoeuvres to relax (lengthen) ASM. In healthy airways, tidal breathing and DI favour cross-bridge detachment of actin and myosin filaments, thus slowing and ultimately limiting ASM shortening. If the ASM layer were to become stiffer, the effectiveness of this tidal stretch would diminish which could lead to excessive airway narrowing (Fredberg, Inouye et al. 1999). This may occur in the remodelled airway wall where a greater muscle mass or restricted movement of the ASM layer may increase wall stiffness. For the same applied force, a stiffer ASM stretches less and muscle contraction is maintained, which in turn promotes greater airway stiffness and less strain with each subsequent manoeuvre. As the ASM layer becomes stiffer, fewer action/myosin bonds un latch causing the ASM layer to narrow more. This vicious cycle causes the smooth muscle to effectively “freeze” in a latched state, becoming so stiff that the DIs can no longer release the myosin binding to cause relaxation (Fredberg 2000; Oliver, Fabry et al. 2007). Additionally, there is evidence to suggest that the cytoskeleton of the smooth muscle may adopt a stiff, solid-like phase when ASM movement is restricted for a period of time, contributing to airway stiffness (Mijailovich, Fredberg et al. 1996). This model therefore proposes that the ASM layer
plays an important role in the ability of the airways to bronchodilate in response to a DI, and may be the most important contributor to the development of airway hyperresponsiveness.

The understanding of airway narrowing and the underlying mechanisms has evolved over time (Oliver, Fabry et al. 2007). ASM contraction should not be modelled by static mass-force relationships (Figure 1.19A, B) but rather must consider the muscle’s natural movements during breathing (Figures 1.20). Thickening of the airway wall, increased ASM mass and loss of parenchymal attachments have all been implicated in excessive airway narrowing. It has now been shown that an increased ASM mass and stiffness has the potential to dominate the mechanical response of airway narrowing. We propose that changes to the structure and function of the ASM layer are the most important contributors to airway narrowing in airway disease.

Figure 1.19: Old views on ASM contraction and airway narrowing A and B (adapted from Oliver, Fabry et al. 2007).

Figure 1.20: New views on ASM contraction and airway narrowing (adapted from Oliver, Fabry et al. 2007).
1.8 The Airway Smooth Muscle (ASM) Layer in Airway Disease

Increased thickness of the ASM layer is a prominent feature of airway remodelling in asthma and COPD and contributes most to the increased airway wall thickness (James and Wenzel 2007). Mathematical models suggest that ASM thickening is also a primary contributor to the airway hyperresponsiveness (excessive airway narrowing) in asthma (Oliver, Fabry et al. 2007). The ASM layer is composed of ASM cells, ECM and occasional blood vessels and inflammatory cells. In sections of airways, fixed and stained for microscopic examination, spaces are observed between ECM and ASM cells, presumably due to preparation artefacts, although tissue oedema could have the same affect since it is not preserved in processes that require tissue dehydration. The mechanisms responsible for the increase in the ASM layer thickness are generally considered to be either an increase in the number of smooth muscle cells (hyperplasia) or an increase in the volume of the individual smooth muscle cells (hypertrophy) or a combination of both. Variations in the contribution of ECM, other structures or cells or fixation artefact may not only confound estimates of the volumes but also contribute to the total volume of the ASM layer and later its mechanical properties.

Distinct pathways may lead to ASM hypertrophy or hyperplasia (Bentley and Hershenson 2008) or to an increased deposition of ECM (Dekkers, Maarsingh et al. 2009). Hypertrophy and hyperplasia of ASM cells may increase the ability of the ASM to shorten by increasing the total contractile proteins present in the ASM layer. With more contractile proteins, more force development may result, contributing to excessive airway narrowing as seen in asthma and COPD. Other changes to the ASM layer, such as changes in the volume or composition of the ECM, may also affect the movement of the smooth muscle by altering the loads (within the ASM layer) that oppose shortening. The ECM may become more compliant reducing the resistance to the ASM shortening, or more rigid which could hold the ASM in a contracted state and prevent it from re-lengthening. Whether ASM hypertrophy or hyperplasia plays a definitive role in the thickening of the ASM layer in asthma and COPD, and the contribution of ECM to the ASM layer is unclear. The presence of ASM hypertrophy and hyperplasia and changes in the ECM in small compared with large airways, and the impact of disease severity and duration on ASM remodelling is also unclear. Further investigation of these factors may influence the development of appropriate treatment strategies and therapies targeting remodelling of the ASM layer in airway disease.
1.9 The ASM Layer in Asthma

Since the studies of Heard and Hossain in the early 1970s (Heard and Hossain 1973), investigators have assessed the relative contribution of hypertrophy and hyperplasia to the increased thickness of the ASM layer in asthma. A number of studies have shown that the increased thickness of the ASM layer in asthma involves smooth muscle hyperplasia (Ebina, Takahashi et al. 1993; Woodruff, Dolganov et al. 2004) and there is additional evidence that hypertrophy also contributes (Ebina, Takahashi et al. 1993; Regamey, Ochs et al. 2008). Studies such as these are difficult since they require access to large sections of airway wall from small and large human airways and the applied counting techniques are labour intensive. Nevertheless, in recent years there has been advancement in knowledge on the factors that contribute to ASM thickening, especially with the more widespread application of stereological techniques and principles. Several important studies that have adopted stereological techniques to examine structural changes to the ASM in asthma are discussed below and include a detailed review of the methods used. Summarised findings and conclusions are shown in italics.

1.9.1 Human Studies

Heard and Hossain (Heard and Hossain 1973) examined central airways from the lungs of five males who had suffered asthma for at least ten years before death. The whole left lung was removed and fixed in formaldehyde at a pressure of 25-30cm for a minimum of 72 hours. The bronchi from the basal segment to the posterior basal segment were examined in all patients. Resected segments of these bronchi were embedded in paraffin and systematically sampled from the proximal to the distal end. Serial sections were cut at 10µm thickness and every tenth section was mounted and stained with haematoxylin and eosin (H&E) to provide semi-serial sections spanning 2mm along the bronchus. The sections were each magnified, projected onto a screen, and point counting was performed to estimate the volume of the ASM layer along the 2mm length of airway. They found that the volume of the ASM layer was approximately three times greater in asthmatic subjects compared with controls, and the mean number of cell nuclei was similarly increased. From these results, Heard and Hossain concluded that hyperplasia, rather than hypertrophy, accounted for the increased bronchial muscle volume seen in these cases of fatal asthma.
Ebina and coworkers (Ebina, Yaegashi et al. 1990), examined autopsy lungs (formalin fixed) from 16 asthmatics and 20 control subjects free from bronchopulmonary disease, as well as 13 non-asthmatic patients with COPD. The non-asthmatics had no history of chronic lung disease and were matched to the mean age of the patients with asthma. All asthmatic patients were over 27 years of age at the onset of asthma and had “therapy-resistant” bronchial asthma. Patients had been treated with corticosteroids, theophylline, bronchodilators or other drugs, and were steroid dependent. Subjects with asthma either died due to an asthma attack or “side effects related to the long term use of oral corticosteroids”. Tissue blocks were taken to include multiple large bronchi, small bronchi and bronchioles extending from the segmental bronchi to the terminal bronchioles. Blocks were embedded in paraffin, sectioned at 3μm thickness and stained with elastica-Goldner for visualisation of ASM nuclei. The perimeter of the basement membrane (Pbm) and the cross-sectional area of the ASM layer were measured by tracing on a digitiser and the anatomic radius and mean thickness of the muscle layer were calculated. The relationship between the thickness of the muscle layer and the size of the airway were examined and two distinct patterns of smooth muscle distribution emerged. Compared with controls, two ‘asthmatic groups were identified: group one had increased thickness of the ASM layer only in the central airways (which the authors termed “Type I”); and the second group had increased thickness of the ASM layer in both the central and peripheral airways (termed “Type II”).

In a subsequent study (Ebina, Takahashi et al. 1993), uninflated lungs from ten asthmatics and ten control subjects were examined, as described above. Tissue specimens were taken from the segmental bronchi to the terminal bronchioles and microscopically examined to ensure they contained airways of various dimensions. The specimens were embedded in paraffin, sectioned at 3μm thickness, stained with elastica-Goldner and used to determine the presence of intact cross-sectional airways, necessary for 3D sampling of smooth muscle cells. Once suitable airways were found, the paraffin blocks were trimmed and re-embedded before 100 - 200 serial sections were cut (2μm thick) and stained with H&E to count ASM nuclei. Sampling of the ASM was performed using a modified physical disector method in which serial sections were stacked to produce a sampling space of 40 - 60μm thick. The area of smooth muscle and the contours of the nuclei were delineated by projecting ASM onto sheets of tracing paper, and the tracings repeated for consecutive tissue sections. Nuclei, usually emerging over several successive sections, were identifiable by illuminating the stack of
tracing paper from behind. By sampling the cells within the stack in this way, an unbiased numerical density of ASM was obtained. The number of cells in a unit volume was given by $N_V = N_S/V_S$ where $N_S$ is the number of cells contained in a stack and $V_S$ is the volume of the stack. *The authors found that both Type I and Type II asthmatics had ASM cell hyperplasia in large bronchi, but to a different degree. Hypertrophy was seen only in Type II asthmatics and involved both large and small airways, suggesting different mechanisms of ASM remodelling between the Type I and Type II asthmatics.* *The authors could not find differences in airway pathology or subject characteristics to account for these differences.*

Kuwano *et al.* (Kuwano, Bosken *et al.* 1993) used cross-sections of peripheral airways to compare the dimensions of the airway wall internal and external to the ASM layer, in subjects with asthma or COPD and those with normal lung function. Tissue from 15 asthmatic subjects (mild and moderate), 15 subjects with mild COPD (FEV$_1$% pred <85) and 15 patients with normal lung function were obtained from post-mortem specimens or post-operatively after lung resection for peripheral tumours. Fixed tissues were embedded in paraffin, sectioned at 5μm and stained with the Masson’s Trichrome technique or with H&E. All small airway cross-sections with a long-short diameter of 3:1 or less were examined. Airways containing cartilage and respiratory bronchioles were excluded from the study. On an average of five airways per case, the dimensions of the airways, the area of smooth muscle and the area of bronchial blood vessels were measured by tracing the microscopic images onto a digitising screen. Comparisons between cases were made on airways with a similar range of sizes, determined by the perimeter of the basement membrane. *They found that in subjects with asthma, the total wall area, inner wall area, outer wall area and smooth muscle area were increased compared with controls. These parameters were also increased in subjects with fatal asthma compared with non-fatal asthma. The area of the ASM layer was found to be two to three times thicker in asthmatic airways compared with controls, although the contribution of hypertrophy or hyperplasia to this observation was not assessed.*

Benayoun and colleagues (Benayoun, Druilhe *et al.* 2003) used endobronchial biopsies to study several features of airway remodelling and inflammation in asthma. Six biopsies were taken from the subcarinae of the right middle lobe and right lower lobe in forty subjects with asthma of varying clinical severity and ten non-asthmatic control subjects. Using 5μm haematoxylin stained sections morphometry was used to assess
total biopsy areas, subepithelial basement membrane thickness, epithelial integrity and area of submucosa occupied by the ASM and mucus glands. The thickness of the ASM layer was evaluated by measuring the distance between the subepithelial basement membrane and ASM at regular intervals over 50µm around the luminal perimeter, at a minimum of 20 sites. The diameter of ASM cells measured across the mid-point of the nucleus was also measured in at least two random fields for each tissue section for a minimum of 50 cells. ASM cell diameter was used as a marker of cell size. Results showed that the thickness of the ASM layer increased with the severity of asthma, with a significant decrease in the distance between the basement membrane and the ASM layer seen in subjects with severe persistent asthma. ASM cell diameter increased by 1.5-fold in patients with mild to moderate asthma, and a 3-fold increase was seen in patients with severe asthma compared with control subjects. Benayoun and colleagues concluded that ASM hypertrophy, rather than hyperplasia, was a marker of asthma severity. Although these results agree with previously published results, the effects of ASM shortening were not taken into account in this study. As ASM cells shorten along their long axis, their transverse axis must increase if tissue volume remains constant. As a single linear measurement was used as an estimate of ASM cell size, the movement of ASM cells prior to fixation, and thus their shortened or lengthened state, may have affected the estimation of ASM size.

Woodruff and co-workers (Woodruff, Dolganov et al. 2004) studied 14 healthy controls and 14 patients with mild asthma to quantify smooth muscle cell parameters. Asthmatic subjects were doctor-diagnosed, used only inhaled β-agonist medications and had documented bronchial hyperresponsiveness. Non-asthmatic subjects were required to have a provocative concentration of methacholine causing a 20% decrease in FEV₁ (PC₂₀) of greater than 16mg/ml during methacholine challenge. Approximately six bronchial biopsies were taken from the second to the fourth carina, fixed, and serial sections were cut at 3µm thickness. Stereological software was used to measure ASM cell number and size by counting ASM nuclei in airway sections a known distance apart – the physical disector method (Nyengaard and Gundersen 1992). Using this approach and stereology software, systematic random sampling was achieved and point counting applied to facilitate the measurement ASM nuclei. The number of points falling on smooth muscle and the number of lines intersecting the epithelial basal lamina were recorded to allow calculation of the volume fraction of smooth muscle per surface area of basal lamina. A mean of 89 fields were measured over five biopsies with
approximately 200 points counted per subject. ASM cell number was also measured using the physical disector technique and point counting, on paired serial sections. The investigators found no increase in cell size, although cell number was 2-fold higher in asthmatic subjects compared with control subjects. As in previous studies, the volume fractions of ECM and other elements within the ASM layer were not assessed.

There is evidence to suggest that airway remodelling is present early in the clinical course of the disease. Regamey et al. (Regamey, Ochs et al. 2008) studied endobronchial biopsies from school age children (8 - 14 years) with chronic inflammatory lung diseases to determine whether increased ASM mass is already present prior to adulthood. They examined 24 cases of asthma, 27 with cystic fibrosis, 16 with non-cystic fibrosis bronchiectasis and 11 control children without lower respiratory disease. Up to five biopsies were taken from the subsegmental bronchi of the right lower lobe in each child and processed into paraffin wax blocks. For each case, the ASM volume fraction was measured using point counting on 3μm sections stained with H&E. In a subset of children (12 asthma, ten cystic fibrosis, nine bronchiectasis and five controls), ASM number and mean cell volume were measured using the physical disector technique on paired serial sections. They found that the ASM volume of children with chronic inflammatory lung disease was significantly greater than that in control children. They also found a significant relationship between the volume fraction of ASM in the subepithelial tissue and the amount of improvement in FEV₁ after bronchodilator inhalation in the group with asthma. There was a trend to increased ASM cell size (hypertrophy) and number (hyperplasia) in each disease group although the increased ASM number was only statistically significant for the asthma group. The lack of significance in the other groups may partly be due to the smaller number of biopsies used to study ASM number. As endobronchial biopsy samples are limited in size, structural features available for morphology vary between subjects and are only a reflection of the ASM mucosa rather than the entire airway wall thickness. A larger number of biopsies per subject should ideally be sampled to account for this structural variability. Additionally, the location of the site of biopsy collection (at the subcarinae rather than from the lateral walls), may not represent the structure of the entire conducting airways and should be considered when interpreting results. Despite the limitations, this was the first quantitative study to show an increased ASM mass in chronic airway disease in children. The findings of ASM hyperplasia in children with asthma are of a similar magnitude to the remodelling changes observed in adults.
suggesting that structural changes to ASM appear early in the course of chronic inflammatory airway diseases.

1.9.2 Animal Models of Asthma

Utilising animals to study human conditions is made possible by the highly conserved nature of the processes regulating lung function, inflammation and respiration amongst most mammalian species. Animal models have been used to examine ASM cell hypertrophy and hyperplasia under a number of experimental conditions. The BALB/c mouse model has been used extensively since it can be sensitised to allergen and produces a type 2 allergic response characterised by eosinophilic inflammation of the airways. BALB/c mice, sensitised and exposed to ovalbumin, demonstrate features of airway remodelling including thickening of the airway smooth muscle layer (Bentley, Deng et al. 2009). Other animal models that have been used in the study of asthma and COPD include rat, guinea pig, rabbit, cat, dog, sheep, pig, horses and primates with rodent models the most commonly used (Allen, Bischof et al. 2009; Canning 2003; Mitchell, Turner et al. 2004). Although hyperplasia is reported in several studies using animal models (Herszberg, Ramos-Barbon et al. 2006; Bentley, Deng et al. 2009), the term has been used to somewhat inaccurately describe the increased thickness of the ASM layer in general, and in many studies it is unclear whether there is indeed an increase in cell number. Fewer animal studies have specifically examined the relative contribution of ASM cell hypertrophy and hyperplasia to the thickening of the smooth muscle layer and the underlying mechanisms.

De et al. (De, Zelazny et al. 1995) studied guinea pig ASM cells in culture to determine whether specific inflammatory cytokines, present in humans with asthma, are responsible for causing ASM hypertrophy or hyperplasia. The entire trachea from normal male guinea pigs was used to obtain smooth muscle cells which were cultured for several weeks until they reached confluence. Morphometry of cell appearance and shape was used to determine whether the cultured cells still represented pure ASM. Cell cultures were then treated with interleukin (IL)-1β or IL-6 and examined one, three and five days following cytokine exposure. ASM cell hyperplasia and hypertrophy were assessed directly through cell counting, and by determination of total DNA content. They found that both IL-1β and IL-6 induced significant ASM cell hyperplasia in a concentration-dependant manner. Additionally, both cytokines increased the total DNA content of ASM cells, which may reflect ASM hypertrophy. The authors note that one
limitation to their study was in obtaining ASM cells for culture exclusively from tracheal tissue, and that anatomic location may influence whether ASM cells become hypertrophic or hyperplastic. In the study by Ebina et al. (Ebina, Yaegashi et al. 1990), two asthmatic phenotypes were described, one group displaying central airway hyperplasia, the other displaying both hyperplasia and hypertrophy in central airways and hypertrophy in peripheral airways. Therefore the observed effect of IL-1β and IL-6 on ASM cells may have been different if ASM cells were isolated from intraparenchymal bronchi rather than from the trachea.

Whole lungs from BALB/c mice were used by Bentley et al. (Bentley, Deng et al. 2009) to examine the role of glycogen synthase kinase-3β (GSK3β) on hypertrophy or hyperplasia of ASM. GSK3β is a constitutively active kinase that has been shown to negatively regulate transcription factors involved in muscle specific gene expression. The mice were sensitised to sterile ovalbumin by intraperitoneal injection and repeatedly challenged over a month with intranasal instillations of ovalbumin. Lungs from age and size matched control mice (treated with phosphate buffered saline) and six ovalbumin sensitised mice, were sectioned into 62 and 80 slides respectively. Stereological methods were used to examine the number and volume of ASM cells expressing alpha-smooth muscle actin and phosphorylated GSK3β. They found that OVA treatment mice to show a 3-fold increase in ASM volume density, a 1.5-fold increase in ASM number per unit volume (Nᵥ), and almost a 60% increase in volume per cell (Vᵥ/Nᵥ). The volume density of pGSK was also increased by 12-fold and the Nᵥ and Vᵥ/Nᵥ of pGSK were also increased (5x and 1.6x respectively). From these results it was concluded that both hyperplasia and hypertrophy are involved in the increased thickness of the ASM layer in their ovalbumin treated mouse model. Further, phosphorylation and inactivation of GSK3β was associated with ASM hypertrophy and hyperplasia in OVA treated mice, and may represent a potential pathway to airway remodelling in asthma.

The Brown Norway rat is another animal that has been extensively used which, when sensitised to allergen, displays features of airway remodelling as seen in asthma including airway hyperresponsiveness, airway inflammation and increased area of the ASM layer in cross section (Pini, Torregiani et al. 2006; Xu, Vlahos et al. 2002). This model was used by Moir et al. 2003 (Moir, Leung et al. 2003) to measure bronchiolar smooth muscle content, cell number and tension development in five control rate (saline
treated) and five rats sensitised to ovalbumin (intraperitoneal injections over three days). Rats were then challenged for three days starting on day six, for a total of six 15 minute exposures. Bronchial segments from the distal subsegmental bronchus of the right lung were dissected free of parenchyma and used for assessment of airway wall morphometry. Three to seven 5µm transverse airway sections were used to determine smooth muscle content, by measuring the area occupied by the ASM and the perimeter of the basement membrane. The numbers of ASM nuclei were also counted and both measurements were normalised for airway size. The authors found that ovalbumin challenged rats had a 1.4-fold increase in ASM area compared with the saline challenged rats; however the numbers of ASM nuclei were not different between groups. The increased ASM area, without an increase in cell number, suggests that hypertrophy, rather than hyperplasia, accounts for the increased ASM content displayed by ovalbumin challenged rats. This increase in ASM mass was also associated with increased maximal tension development of bronchial segments in vitro.

Heaves is a naturally occurring asthma-like disease in horses characterised by airway inflammation, bronchoconstriction and remodelling (Herszberg, Ramos-Barbon et al. 2006; Leclere, Lavoie-Lamoureux et al. 2011; Lowell 1964). Exacerbations are caused when susceptible horses inhale environmental antigen and are associated with cough, wheeze and exercise intolerance. These symptoms can be controlled in a similar manner to asthma in humans by antigen avoidance or administration of corticosteroids and bronchodilators. Leclere et al. (Leclere, Lavoie-Lamoureux et al. 2012) used 11 heave-affected horses to examine the long-term effects of antigen avoidance and inhaled corticosteroids on airway remodelling. Horses were exposed to hay until they developed symptoms associated with airflow limitation. Five horses were turned out to pasture for strict antigen avoidance. The remaining six horses were treated with inhaled corticosteroids and remained exposed to hay for six months before also being turned out to pasture. Lung function, bronchoalveolar lavage, and peripheral lung biopsies were taken one month, six months and 12 months after treatment in both groups of horses. ASM area, number of ASM nuclei and internal airway perimeter were measured on cross sectional airway sections from 5–17 airways per horse. They found ASM area to decrease by 30% over a 12 month period, in both groups of horses, with this decrease occurring faster in horses treated with inhaled corticosteroids. The number of ASM nuclei corrected for airway size was also shown to decrease in the corticosteroids treated group at 12 months post-treatment. These data suggest that ASM hyperplasia is
responsible for the increased ASM mass observed in horses with heaves. Although ASM hypertrophy was not directly measured, and so cannot be ruled out, the observed hyperplasia is consistent with some human studies (Woodruff, Dolganov et al. 2004). The reduction in ASM hypertrophy with treatment also suggests that extended inhaled corticosteroid use may be effective in offsetting ASM remodelling.

Transforming growth factor alpha (TGFα) and epidermal growth factor receptors are associated with airway remodelling and there is increased expression of both the ligand and receptor in the airways of subjects with asthma (Aubert, Dalal et al. 1994). In mice, TGFα induces airway fibrosis, pleural thickening, pulmonary hypertension and increased expression of early growth response-1 (Egr-1) (Kenyon, Ward et al. 2003). The EGR receptor has been shown to stimulate or repress gene transcription which can result on promotion or suppression of cell growth as seen in studies of tumours (Levin, Casey et al. 1994), (Huang, Fan et al. 1997). Kramer et al. (Kramer, Mushaben et al. 2009) used TGFα transgenic mice mated with Egr-1 knockout mice to determine the role of Egr-1 in TGFα induced lung disease. The mating protocol produced three different genotypes: mice that were Egr-1 (Egr-1\(^+/+\) i.e. wild type), Egr-1\(^+-\) and Egr-1\(^-/-\) (i.e. Egr-1 knockout mice). Mice from each genetic variant were placed on doxycycline for ten days, three or eight weeks to activate the expression of TGFα (via a conditional doxycycline regulated transgene). Control transgenic mice (Egr-1\(^+/+\), Egr-1\(^+-\) and Egr-1\(^-/-\) not exposed to doxycycline were also examined. Lungs were fixed and 5\(\mu\)m sections were stained for Egr-1, Ki67, alpha-smooth muscle actin and smooth muscle II myosin heavy chain using immunohistochemistry. They found mice with activated TGFα expression produced thickening of the ASM layer, cell proliferation (identified by Ki76) and increased expression of Egr-1 compared with controls. The ASM area, as identified by alpha-smooth muscle actin and smooth muscle II myosin heavy chain staining, was 1.7-fold greater in Egr-1 knockout compared with Egr-1\(^+/+\) mice and two-fold greater than that observed in control mice. The number ASM cells was also increased by 1.8 times in Egr-1 knockout mice compared with Egr-1\(^+/+\) mice, however the ASM cell size showed no differences with either doxycycline treatment or genotype. These data show the lack of Egr-1 increases the severity of TGFα induced airway disease, and that ASM cell hyperplasia, rather than hypertrophy, to be the prominent cause of the increased ASM area in the Egr-1 knockout mice. Mice were also shown to exhibit pronounced airway narrowing to bronchoconstrictor challenge, representing a realistic model of airway hyperresponsiveness, as displayed by severe asthmatics.
While the value of studying a human disease using an animal can be debated, these animal studies have been helpful in demonstrating that both hypertrophy and hyperplasia of ASM may occur under different experimental conditions designed to simulate human airway disease. In particular, animal models have helped to show that different mechanisms are involved in the development of airway disease and that increased ASM size and number is associated with abnormal airway function.

1.10 The ASM Layer in COPD

1.10.1 Defining COPD

Numerous terms have been used in the past to describe the condition of fixed or irreversible airflow obstruction including chronic airflow obstruction (CAO), chronic obstructive lung disease (COLD), chronic airflow limitation (CAL) and chronic non-specific lung disease (CNSLD). Under the auspices of the GOLD initiative (Rabe, Hurd et al. 2007) the term chronic obstructive pulmonary disease (COPD) has been most commonly adopted worldwide to describe irreversible airflow obstruction. As discussed in Section 1.6, COPD is defined using GOLD criteria as a post-bronchodilator (e.g. 200µgm salbutamol) FEV₁/FVC ratio of <0.7 and an FEV₁ <80% of the predicted value, with severity categorised into GOLD Stages based on levels of FEV₁ relative to the predicted value. Debate continues about the effects of age on these variables, the definition of normal and which predicted values are most appropriate for individuals or populations. In most population studies, sex, height, age and race are the most important subject variables that determine the FEV₁. Using GOLD criteria, the prevalence of COPD has been assessed globally using a standard protocol as part of the burden of obstructive lung disease (BOLD) studies and has included Australia (McDonald and Glasgow 2013; Toelle, Xuan et al. 2013). Internationally the biggest risk factors for COPD are smoking cigarettes and the burning of biomass fuels (Mannino and Buist 2007).

Defining COPD based on spirometry has its limitations. A normal FEV₁/FVC ratio does not exclude airflow obstruction, the FEV₁/FVC ratio declines with age and a low FEV₁/FVC ratio may be observed in subjects with normal but large lungs (high FVC). Normal spirometry may also be observed in the presence of considerable disease related to smoking, particularly emphysema. The pathology that contributes to airflow limitation in COPD involves both the lung parenchyma and the airways, but are likely
due to separate, genetically determined responses to cigarette smoke exposure (Patel, Make et al. 2006). Interactions between the lung parenchyma and the airways are both necessary in order to drive air in and out of the lungs. Abnormalities of one or both of these structures can result in airflow limitation. Parenchymal disease, such as emphysema, reduces lung elastic recoil which decreases the driving force of air from the lungs. Similarly, loss of attachments between the airway and the parenchyma may result in airway collapse as the support which helps maintain airway patency during expiration is lost. Changes to the airways can be caused by airway remodelling and inflammation and can result in increased thickness of the compartments of the airway wall. Increased airway wall thickness can result in excessive narrowing and increased resistance to airflow though the airways. Both the small airways and the large airways have been shown to have pathological abnormalities in COPD (Chung 2005; Jeffery 2001). It has been recognised that “early” disease, especially inflammation (which might result in scarring and airway narrowing) can go undetected in the small airways before abnormalities of FEV\textsubscript{1} or total airway resistance are observed (Pedley, Schroter et al. 1970; Hogg, Macklem et al. 1968). This has led researchers to focus on the physiology and pathophysiology of the small airways with the aim of trying to detect and treat preclinical small airway disease.

1.10.2 The Role of the Small Airways

Due to their large combined cross-sectional area, the small airways contribute little to total airway resistance in normal lungs (Weibel 1963). In healthy subjects, less than 10% of total airway resistance (below the larynx) can be attributed to the small airways (<2mm in diameter) (Hogg, Macklem et al. 1968). In COPD, the resistance of the lungs is approximately doubled and this largely involves structural changes to the small airways (Hogg, Macklem et al. 1968; Van Brabandt, Cauberghs et al. 1983; Yanai, Sekizawa et al. 1992). Changes to the small airways which will increase total airway resistance can occur in two ways. Removal of half of the existing airways would achieve a doubling in small airway resistance. Alternately, generalised narrowing of all peripheral airways will increase airway resistance which is proportionate to the radius of the lumen to the fourth power (Hogg, Macklem et al. 1968; Hogg, McDonough et al. 2009). In COPD subjects, the increase in peripheral airway resistance has been reported to be anywhere from four to 40 times greater than normal subjects (Hogg, Macklem et al. 1968). Due to this large increase, the airway resistance of COPD subjects is best explained by both the destruction and narrowing of small airways.
1.10.3 Small Airway Destruction

The loss of small airways in COPD has been observed in numerous studies (Hogg, Macklem et al. 1968; Matsuba and Thurlbeck 1972; Hogg, McDonough et al. 2009; Diaz, Valim et al. 2010). Obliteration of the small airways in COPD was first reported by Hogg and colleagues in a post-mortem study of airflow and airway structure (Hogg, Macklem et al. 1968). They measured airway resistance, emphysema score and airway dimensions in the lungs of subjects with COPD and compared them to normal lungs. A subsequent study by Hogg et al. (Hogg, McDonough et al. 2009) extended these findings on the pathology of small airways by using micro-CT. Micro-CT uses a micro-focused X-ray source and X-ray detectors to measure the density of the lungs, and was used to acquire high-resolution images of the bronchiolar and alveolar microstructure in explanted lungs of subjects with COPD. The number and cross-sectional area of the terminal bronchioles and alveoli were examined in transplant lungs from four subjects with very severe COPD (GOLD Stage IV) and compared with four healthy donor lungs. Lungs were prepared by inflating to total lung capacity and freezing in liquid nitrogen. The frozen lungs were then cut into 2cm thick slices and cores of lung parenchyma were fixed for examination using micro-CT. Image analysis software was used to measure the dimensions of the alveoli and the number and dimensions of the terminal bronchioles present in these lungs. Lungs from the severe COPD subjects were found to have an approximately ten-fold reduction in the number of terminal bronchioles compared with controls. The cross-sectional areas of the remaining terminal bronchioles were also found to be 100-fold lower in COPD subjects, with the changes to the bronchioles being observed in both healthy and emphysematous regions of the lung. These findings have recently been replicated in a similar study of a large number of COPD cases showing the number of terminal bronchioles to decrease as COPD severity worsens (McDonough, Yuan et al. 2011). The fact that loss of terminal bronchioles and reduction in cross-sectional area occur in the healthy parts (non-emphysematous) of the lung suggests that these changes occur prior to the onset of emphysematous lung destruction. Whether this loss of airways occurs as part of the same destructive process of emphysema is currently unknown. Progression of these changes may be responsible for the steady decline in lung function over time, leading to increasing severity of COPD.

1.10.4 Airway Remodelling in COPD

As well as airway loss, remodelling of the small airways also occurs, with the nature and magnitude of this remodelling dependent on the severity of COPD. Hogg et al.
(Hogg, Chu et al. 2004) studied markers of airway remodelling and inflammation in 159 subjects with varying severities of airflow obstruction (GOLD stages I-IV). Samples of fixed, processed tissue were cut into 5μm thick sections and stained separately to identify inflammatory cells including neutrophils, eosinophils and macrophages. Digital images of the small airways were also obtained for morphometric analysis to determine the maximal luminal area, airway secretions and airway wall thickness. The airway wall was compartmentalised into the epithelium, lamina propria, smooth muscle and adventitia for measurements of airway dimensions. Results showed that an increase in total airway wall thickness, due to an increase in each of the airway wall compartments was associated with COPD severity. Mucus plugging of the lumen and the extent and severity of the inflammatory response were more weakly associated with disease severity. In the same study, Hogg found that the thickness of the ASM layer in the small airways increased with disease severity. Severe and very severe cases of COPD were found to contain up to 40% more muscle than the mild, moderate and ‘at risk’ groups.

Although significant, this increase in ASM observed by Hogg and other (Hogg, Chu et al. 2004) is less than that observed in cases of asthma, which is up to three times thicker than that of control subjects (Kuwano, Bosken et al. 1993). Significant increases in the area of the layer of the smooth muscle have been reported in other studies of the small airways in patients with COPD (Kuwano, Bosken et al. 1993; Saetta, Di Stefano et al. 1998) with an increase of 20% (range 12-37%) in the area of ASM. Pathological changes such as increased total airway wall thickness, loss of small airways, mucous gland hyperplasia, and subepithelial fibrosis have also been observed in the small (Hogg, Chu et al. 2004; Hogg, Macklem et al. 1968) and large (Chung 2005; Jeffery 2001) airways of subjects with COPD. However the changes in the components of the ASM layer that contribute to the remodelling, in relation to severity of COPD, have not been fully examined.

Saetta et al. (Saetta, Di Stefano et al. 1998) also studied airway remodelling in the small airways of subjects with COPD to determine whether the inflammatory process differed from those of healthy individuals. Lungs were obtained from 16 subjects with COPD and seven smokers with normal FEV$_1$ (controls) who were due to undergo lung resection surgery. Lung function was performed on all subjects prior to surgery. Tissue blocks of subpleural parenchyma (4-6 blocks per subject) were taken from the lobe received at surgery and were fixed in formalin and embedded in paraffin. Serial sections
5µm thick were cut for morphometric and immunohistochemical staining for neutrophils, macrophages and CD4 and CD8\(^+\) T-lymphocytes. Quantification of cellular infiltrates on immunohistochemistry sections was performed within the airway wall (excluding the smooth layer) and results were expressed as number of cells per square millimetre of tissue examined. Morphometric analysis of internal perimeter, total airway wall and the area of ASM were performed on sections stained with H&E. *Histological findings showed no difference in the total wall area between groups, however the area of the ASM layer was significantly increased in subjects with COPD compared with smoking controls. Immunohistochemistry showed the number of CD8\(^+\) T-lymphocytes to be increased in COPD subjects compared with control smokers and showed a negative correlation with FEV\(_1\)% predicted. The inverse relation of increased CD8\(^+\) and reduced lung function suggested a possible role for these cells in the pathogenesis of smoking-induced airflow limitation. No differences were observed in the numbers of neutrophils, macrophages and CD4\(^+\) T-lymphocytes between groups.*

Changes to the small airways in COPD have been well-studied, with fewer studies examining the structural changes of the larger cartilaginous airways. Tiddens *et al.* (Tiddens, Pare *et al.* 1995) examined both the large and small airways in subjects with fixed airflow obstruction to determine whether inflammation of the peripheral airways correlates with airway wall thickness and the area of ASM in large airways. Lungs were obtained from 72 patients with varying severities of COPD undergoing surgery for resection of a peripheral lesion. Twenty-five patients had no significant airflow obstruction (FEV\(_1\)/FVC >75% pred), and 48 subjects were classified as having mild or moderate COPD (FEV\(_1\)/FVC <75% pred). Macroscopically normal cartilaginous airways and blocks of lung parenchyma containing respiratory bronchioles were cut in cross section and embedded in paraffin. Transverse sections (5µm) from 341 cartilaginous airways were stained with Masson’s Trichrome and used for measurements of airway dimensions including the perimeter and area of the basement membrane, the outer border of the smooth muscle and the outer edge of the airway (defined by the outer edge of the adventitia). The areas of smooth muscle and cartilage were measured using an automated image analysis system. The membranous bronchioles were graded from the amount of inflammation, fibrosis and muscle hypertrophy. *The authors found increased thickness of the inner airway wall in large airways compared with small airways and the increased thickness was associated with a reduction in FEV\(_1\)/FVC. The degree of peripheral inflammation was significantly*
related to the inner airway wall area of the large airways of COPD subjects however it was not related to the area of smooth muscle. This study was the first to measure airway dimensions of a large number of cartilaginous airways in subjects with COPD. Although prior studies had examined some large airways (Bosken, Wiggs et al. 1990) their numbers were too few (only 20 large airways examined) to comment on the structural differences between the large and small airways.

Few studies have directly assessed whether the increased thickness of the ASM layer in COPD is due to hypertrophy or hyperplasia of the ASM cells. The study of Ebina et al. (Ebina, Yaegashi et al. 1990) used stereology principles to study autopsy lungs from 16 asthmatics, 13 subjects with mild COPD and 20 healthy controls. Ten blocks were taken from each subject and 20-30 sections (3µm thick) were measured ranging in size from large segmental to small terminal bronchioles. Morphometry was used to measure Pbm and cross sectional area of the smooth muscle layer. Destruction of the very small airways due to inflammation was also observed with airways below 1mm radius were reported as “severely destroyed”. The authors reported a mild degree of hypertrophy of ASM cells to be present in COPD but only in the large airways with the small airways remaining within the range of the controls. It is notable that hyperplasia (a prominent and consistent feature in asthma) was not observed. The relative contribution of the extracellular matrix to the volume of the ASM layer was not assessed and it is therefore possible that an increase in the volume fraction of matrix may have contributed to the apparent increase in ASM cell volume (i.e. the volume of ASM cells may have been overestimated) (Section 1.12). Although a number of aspects of airway wall remodelling and inflammation have been rigorously studied, no other studies of ASM cell size and number have been performed in cases of COPD.

1.11 The Extracellular Matrix (ECM)

The ECM of the ASM layer is not just bedding for ASM cells but makes an important contribution to the mechanical properties of the ASM and airway wall under conditions of health and in asthma and COPD. The contractile and non-contractile functions of ASM are strongly determined by its interaction with the surrounding ECM through cell surface signalling and specific receptors (Parameswaran, Willems-Widyastuti et al. 2006). These interactions affect the capacity of the ASM layer to translate force generation into shortening (Bramley, Roberts et al. 1995). The ECM also provides a
series elastic load that opposes ASM shortening (Lambert, Codd et al. 1994) and an increase or decrease in the amount of ECM will alter the loads accordingly (Bramley, Roberts et al. 1995). The ECM also has important effects on growth and proliferation of ASM, promoting the survival, cytokine synthesis, migration, and contraction of human ASM cells (Parameswaran, Willems-Widyastuti et al. 2006). Increased ECM may contribute to the increased volume of the ASM layer in asthma and COPD, which in itself is an important contributor to excessive airway narrowing (Moreno, Hogg et al. 1986; Lambert, Wiggs et al. 1993). While there seems to be a number of mechanisms through which the ECM may influence ASM force development and shortening, and therefore airway narrowing, their relative importance remains unclear. Studying both the type and amount of ECM within the ASM layer may reveal important insights as to the mechanisms involved in airway hyperresponsiveness in asthma and COPD. As discussed previously, if not corrected for, the volume of ECM within the smooth muscle layer will contribute to the estimated volume of individual ASM cells.

The ECM is composed of a large number of different matrix proteins which have a variety of functions in the airway including the maintenance of structural integrity, aiding cellular adhesion and migration, releasing growth factors and cytokines and aiding in fluid balance and osmotic activity. Due to its wide range of functions, a change in the type or amount of ECM could lead to remodelling and changes in lung function. The major lung ECM components include collagens, elastins, proteoglycans, fibronectin, biglycan, lumican, versican, decorin and tenascin (Parameswaran, Willems-Widyastuti et al. 2006). The role and the different subtypes of ECM proteins have been examined in the airways from subjects with asthma and with COPD, however it is unclear whether there is a change in the proportion of ECM within the ASM layer, particularly in COPD.

1.11.1 The ECM in Asthma

In 1996 Thomson et al. (Thomson, Bramley et al. 1996) examined the contribution of the ECM to the ASM layer in asthma. They measured the mechanical properties of airways under load and hypothesised that a reduction in the elastance of the ECM within the smooth muscle layer would result in excessive airway narrowing in airway disease. Macroscopically normal bronchial segments were obtained from five severe asthmatic and two non-asthmatic subjects following lung resection for carcinoma. Lungs from three subjects who had previously died of their asthma were also obtained
for morphological assessment. Muscle strips were dissected from the bronchi of the freshly resected lobes and suspended in an organ bath, fixed at one end and attached to a force transducer at the other. Isotonic and isometric length-tension curves were performed and the percent muscle shortening were determined. Post-mortem fixed bronchial airways were removed and divided axially into arcs of cylindrical airways (Figure 1.21). These were embedded so that the cross-sectional profiles of the smooth muscle fibres were visible within the axis of the airway wall. Blocks were cut 1.5μm thick (toluidine blue-O-stained) and ASM cross-sectional area, tissue width and airway size were measured using planimetry. Point counting of ASM and ECM was performed on ten fields of the ASM layer per section and used to determine the mean proportional area of tissue components per subject. They found that the in vitro response of the airway to shortening was increased in asthmatic (28% shortening) compared with non-asthmatic subjects (10% shortening). No differences were found between the amount of ASM and ECM between groups. The authors noted that the organisational structure of the ASM layer is highly variable and there may have been no discernable difference due to the small numbers of subjects studied. These results suggested that an increase in force generation in the airways of asthmatic subjects may not necessarily be due to an increase in the amount of ASM. The authors attributed the increase in force generation in the ASM from the asthmatic subjects to either increased contractility of ASM cells or a change in the ECM to facilitate greater tension distribution throughout the muscle layer, or a reduction in the internal loads that the ASM must overcome to shorten. It is possible that a decrease in the load provided by the ECM may occur as a consequence of airways inflammation in asthma, resulting in excessive airway narrowing. The results of this study show that determining the composition of the ASM layer is important and that the ECM may contribute to the functional changes (including ASM shortening) observed in subjects with asthma.
Figure 1.21: Airway segments cut in transverse and longitudinal planes. Transverse sections show overlap (1.5μm sections) of the ECM and ASM making these components difficult to discern separately. Longitudinally cut sections show good distinction between the ASM and ECM, however other airway structural features are lost (discussed below).

The study by Thomson et al. (Thomson, Bramley et al. 1996) used a unique technique to accurately determine the composition of the smooth muscle layer in bronchial muscle strips, by cutting the airway sections to view the layer of ASM in cross-section (i.e. longitudinal section of the airway segment). However the application of this approach has limitations. Using the cross-sectional profile of the smooth muscle layer provides a clearer differentiation between the ASM, ECM and other elements but it fails to conserve the structure of the airways, preventing further histological measurements from being made. This technique causes unnecessary use of large amount of tissue, as a long segment of airway (~2mm) must be used in order for an adequate amount of ASM to be visible in cross section. Measuring the muscle in this orientation also causes a loss of relationship between the muscle layer and the other features of the airway wall, providing less information for the examination of structure and function relationships. If the same approach was translated to the study of whole airways, basic morphology of airways, including measurements of airway size, total area of the smooth muscle or ASM cell number could not be performed due to the specific orientation of the tissue required for estimation of ASM fractions. Although measuring the composition of the ASM layer is important, an alternative technique could be used that allows for fractional estimates of the ASM layer while still maintaining airway structure (Section 2.6)
**Bai et al.** (Bai, Cooper et al. 2000) compared structural changes in the airways in “young” (19-23 yrs) and “old” (40-49 yrs) subjects with asthma. They hypothesised that if airway remodelling was related to the duration of asthma, then the airways of older subjects (with a longer duration of asthma) should show more remodelling than those of younger subjects, when matched for disease severity. Post-mortem cases of fatal asthma (14 “young”, 11 “old”) and non-fatal asthma (five “young”, six “old”) were examined. Paraffin-embedded airways were cut at 4µm and stained with Gomori-Trichrome and aldehyde fuschin to allow for accurate distinction of connective tissue and ASM. A maximum of 11 airways from each subject were used to measure airway wall area, Pbm, outer border of the smooth muscle and adventitia, the long and short diameters of the airway lumen and the thickness of subepithelial collagen. The proportion of ASM in the airway wall and within the smooth muscle bundles was quantified by the point counting technique. The degree of airway narrowing was estimated as the ratio of the measured lumen area and the calculated lumen area for a relaxed airway and the degree of lumen obstruction (with mucus, exudate, or debris) was also recorded. *Airways from subjects with fatal asthma were found to have increased wall area compared with non-fatal asthma with a three-fold increase in older compared with younger individuals. Luminal exudate content, subepithelial collagen deposition, and proportion of ECM within smooth muscle bundles were similar between young and old individuals with asthma but were greater than in control subjects without asthma. Despite a trend towards greater ASM in older asthmatic subjects, there were no statistical differences in the amount of ASM between young and old subjects with asthma, suggesting that ASM volume remains fairly constant with age. The degree of airway narrowing was also greater in asthmatic subjects compared with controls, with older individuals displaying more narrowing than younger subjects. These results show that although some elements of airway remodelling are related to age and duration of disease while others such as the amount of ASM or ECM do not change significantly with age. Studies of airway remodelling in children with asthma suggest that the airway structural changes that are characteristic of asthma are present early in life, possibly before the onset of symptoms (Pohunek, Warner et al. 2005; Saglani, Malmstrom et al. 2005).*

A number of studies have examined ECM proteins in airways form subjects with asthma, including ECM specifically within the smooth muscle layer. The study of Pini et al. (Pini, Hamid et al. 2007) found that the proteoglycans biglycan and lumican were increased within the ASM layer in moderate cases of asthma compared with severe
cases of asthma and control subjects. They studied biopsies from six control subjects and 27 cases of asthma of varying clinical severity. A total of four to six biopsy specimens were taken per subject, and fixed 5µm sections were used for immunostaining for biglycan, lumican and decorin and versican. Positive staining of the proteoglycans within the smooth muscle layer was determined by red colouration under bright-field illumination. These were quantified by point counting at 1000x magnification using an eyepiece graticule with a 121-point grid. Proteoglycan within the ASM layer was expressed as a percentage of positive staining overlapping the crosspoints of the grid. The authors found a significantly greater proportion of biglycan and lumican in the smooth muscle layer in cases of moderate asthma compared with more severe cases and control subjects. There were no differences in biglycan and lumican between the severe asthma cases and the controls. There were no significant differences for versican and decorin between any groups. The increased matrix deposition in the moderate asthmatics compared with the more severe cases could potentially reduce airway narrowing by decreasing ASM shortening.

Araujo and colleagues (Araujo, Dolhnikoff et al. 2008) examined the fractional areas of lung ECM elements (collagen, elastic fibres, fibronectin and versican) within the ASM layer in both asthmatic cases and controls. Samples were randomly taken from the central and peripheral airways of fatal (n = 35) and non-fatal (n = 10) cases of asthma and from controls (n = 22) and fixed and sections 5µm thick were stained with H&E. Additional staining with Sirius Red was used to estimate total collagen and the Weigert’s resorcin-fuchsin technique with oxidation to identify elastic fibres. Two large and three small airways were studied by image analysis. The area of the ASM layer was measured manually and the fractional area of antibody staining expressed as percent of total area of the ASM layer. Increased area fractions of elastic fibres were found in fatal cases of asthma compared with non-fatal cases and controls. An increase was also found in the fractional area of fibronectin, and the expression of MMP-9 and MMP-12 in the large airways in fatal asthma cases compared with controls. This study showed an increase in the area fractions of elastic fibres in both the large and small airways in patients with fatal asthma which was not observed in non-fatal asthma or non asthmatic control subjects. The changes in airway function that occur in asthma are closely related to their structure and are likely to depend on the location and contributions of ECM proteins, and their interactions with each other and the surrounding parenchyma and cells. The changes that occur in the ECM in asthma and the location of those changes
within the airway wall may have important consequences for excessive airway narrowing in asthma. The beneficial effects of airway remodelling in asthma have been postulated before (McParland, Macklem et al. 2003), however the evidence in favour of this theory is sparse.

1.1.2 The ECM in COPD

The ECM in COPD has been less well studied. Early studies of COPD sought to quantify scaring and fibrosis seen in the lungs of subjects with COPD (Thurlbeck, Fraser et al. 1965). Wright et al. (Wright, Lawson et al. 1983) investigated the effects of smoking status on lung function and the pathological changes to the peripheral airways in patients scheduled to undergo lung resection (n=97). Patients completed a questionnaire on smoking history and undertook lung function testing to determine their FRC and FEV\textsubscript{1}% predicted. Patients were divided into four groups (non-smokers, current smokers, ex-smokers for <2 years, ex-smokers for >2yrs) depending on their smoking status prior to surgery. The mid-sagittal slices from lungs or lobes obtained following surgery were used to estimate emphysema severity. Six blocks of airway tissue were taken from the medial and lateral slices and processed for histological examination. On 5μm sections, all non-cartilaginous and respiratory bronchioles were scored for evidence of inflammation, fibrosis, mucous gland hyperplasia and ASM area. Ex-smokers had a higher FEV\textsubscript{1}/FVC ratio than current smoker with both groups showing evidence of fixed airflow obstruction (FEV\textsubscript{1} <80% predicted). Current smokers had reduced lung volumes compared with non smokers although the lung volumes of non smokers and ex-smokers were similar. Both current and ex-smokers showed similar increases in goblet cell hyperplasia in the membranous bronchioles and increased inflammation and fibrosis in the respiratory bronchioles compared with non-smokers. Subjects who had stopped smoking showed improved lung function, no differences in structural changes between current and ex-smokers were apparent. The emphysema score was similar in both smokers and ex-smokers but was increased in both groups compared with controls. These results show a relationship between airway remodelling and reduced lung function in subjects with chronic airflow obstruction.

Nagai et al. (Nagai, West et al. 1985) also studied the pathology of small airways and emphysema in subjects with COPD. They examined post-mortem lungs from 48 patients with moderate to severe chronic airflow obstruction. Transverse sections of airways were taken from the main, upper and lower lobe bronchi. Emphysema score
was assessed using blocks of lung parenchymal tissue, sectioned 5μm thick. Airway size, ASM area, cartilage and mucosal glands were measured using planimetry and the severities of airway inflammation were measured using a scoring system on 5μm bronchial sections. The authors found an increased amount of muscle in the bronchioles and a reduced number of bronchioles in subjects with COPD compared with controls. As emphysema score increased, the area of ASM and cartilage in the central airways and eosinophilic inflammation in peripheral airways decreased. Mucous gland size was not related to inflammation in the large or small airways. Although fibrosis was increased in subjects with COPD, no relationships were seen between the degree of fibrosis and measurements of airway narrowing or emphysema. The authors concluded that the large airways respond to irritation by increasing production of mucus and the small airways develop inflammation leading to fibrosis and airway narrowing as well as destruction of small airways and alveolar walls, resulting in emphysema.

Airflow obstruction in COPD is attributed to fibrosis of the small airways and loss of elastin in the alveoli. Black et al. (Black, Ching et al. 2008) studied subjects with COPD to determine whether the reduction in elastic fibres observed in the alveoli also occurred in the small airways. Archived tissue blocks obtained following surgery for lung carcinomas were examined from 13 control subjects and 11 subjects with COPD. Subjects were classified as having COPD if they had a post-bronchodilator FEV\textsubscript{1} of <80% predicted and an FEV\textsubscript{1}/FVC of <0.7. Airway blocks were sectioned at 4μm thickness and stained using elastic van Gieson for visualisation of elastic fibres. The volume fraction of elastic fibres in the airway wall and alveoli and the thickness of the airway wall were determined using point counting by randomly sampling according to clock face positions at 12, 3, 6 and 9. The authors found that the volume fractions of elastin fibres were reduced in COPD subjects compared with control subjects, in both the airway wall (14.6 vs. 25.5%) and the alveoli (18.6 vs. 32.8%). A correlation between the amount of elastic fibres and the FEV\textsubscript{1}% predicted of all subjects was also observed. These findings demonstrated a relationship between changes in the airways and alveoli in COPD and suggested that a reduction in elastin fibres may be important in determining the severity of airway disease. A decrease in elastic fibres could contribute to reduced lung function by reducing lung elastic recoil pressure and increasing airway resistance due to loss of alveolar support. Loss of elastic fibres in the small airways may affect the properties of the airways themselves, causing them to narrow excessively or more easily with ASM contraction (Gray and Mitchell 1996).
The degradation of elastic fibres in COPD has also been explored in other studies. Maclay et al. (Maclay, McAllister et al. 2012) investigated whether the reduced elastin in the airways of COPD subjects was due to localised pulmonary or systemic elastin degradation. They examined elastin degradation in the skin from 16 people with varying severities of COPD (GOLD stage I - IV) and from 15 age-matched control subjects. Skin biopsies from sun-exposed and non-exposed areas, and the area of skin covered by elastin fibres were quantified on 3µm sections (elastic van Gieson). Elastin fibres thicken as they degrade, thus samples with a greater area of elastin represent a greater degree of degradation. The expression of MMP -2, -9 and -12 on skin samples was also determined. Elastin degradation in the skin samples was found to be greater in COPD patients in both the sun-exposed (43.6%) and non-exposed (22.4%) compared with controls (26.3% sun-exposed, 18.1% non-exposed). Elastin degradation also correlated with the degree of emphysema in COPD subjects in both sun-exposed and non-exposed skin samples. Expression of MMP-2 and -9 were higher in sun-exposed COPD subjects and were positively associated with elastin degradation. Increased expression of MMPs has previously been shown in patients with COPD and is associated with emphysema (Baraldo, Bazzan et al. 2007). The expression of MMP-9 outside the lung and the increased elastin degradation in the skin suggests that elastin degradation may be a systemic feature of COPD, not just restricted to subjects with emphysema.

Previous studies of COPD have shown an increased thickness of the airway wall and increased fibrosis, however the underlying cause is unknown. Kranenburg et al. (Kranenburg, Willems-Widyastuti et al. 2006) hypothesised that an alteration in the content of ECM proteins in the various compartments of the airway wall accounted for increased wall thickness. To address their hypothesis, they studied the distribution of ECM markers within the basement membrane, lamina propria, bronchial adventitia, and ASM of smokers and ex-smokers with and without COPD. Bronchial tissue specimens were obtained from both current and ex-smokers who had undergone surgery for lung cancer. Subjects were categorised as COPD (n=15) and non-COPD (n=16) with COPD defined as a pre-bronchodilator FEV₁ of <75% predicted, an FEV₁/FVC ratio <0.75 or a change in FEV₁ of <12% following bronchodilator administration. Bronchial airways located away from the tumour site were dissected out and fixed and serial sections were cut (4µm thick) and stained to determine total collagen content and expression of the ECM proteins collagen I, III, and IV, fibronectin, and laminin-β2. Semi-quantitative analysis was performed using a 4 point scoring system of stain intensity ranging from
no stain to very intense staining. The expression of collagens I, III, and IV, fibronectin and laminin were increased in the bronchi of COPD subjects compared with control subjects. ECM proteins studied were significantly increased at sites of epithelial damage in all subjects. Total collagen content and expression of fibronectin and collagens I and III were greater in COPD subjects compared with control subjects. Laminin staining was increased 1.5-fold in the ASM layer of COPD subjects and collagen content in the basement membrane and laminin in the ASM were inversely related to FEV₁% predicted. These findings suggest that the deposition of ECM components in the airways is altered in COPD and contributes to the reduced lung function and airway remodelling observed in COPD.

Studies of subjects with COPD have shown changes in ECM in their airways although how these changes affect the development of disease and the severity of airway narrowing is not clear. Lofdahl et al. (Lofdahl, Kaarteenaho et al. 2011) studied the expression of tenascin-C and alpha-smooth muscle actin in the large airways of subjects with COPD and correlated them with smoking or development of disease. Endobronchial airway biopsies from 20 subjects with COPD, 13 smoking subjects without COPD and 14 non-smoking controls were examined. Four to six biopsies were taken from each subject, from the lobar or segmental carinae of the upper left lobe or the apical segment of the lower left lobe and sections 1-2µm thick were stained by immunohistochemistry. Expression of tenascin-C was quantified, based on stain locations as: within the epithelial cells and basement membrane; in the stroma beneath the basement membrane or; in the connective tissue of the bronchial wall. Myofibroblasts were quantified by the numbers of spindle-shaped cells stained with alpha smooth muscle actin: no cells; 1-4 cells; 5-10 cells or; >10 cells using the same location parameters as tenacin-C. Expression of alpha smooth muscle actin within the ASM or blood vessels was not assessed. Results showed that expression of tenascin-C was greater in the epithelial cells and basement membrane of subjects with COPD. Alpha smooth muscle actin expression was also greater in COPD patients (83%) compared with non-COPD smokers (46%) and non-smokers (41%). No relationships between lung function and tenascin or alpha smooth muscle actin expression were observed. These findings show that airway remodelling occurs in the large airways of subjects with COPD, a finding that agrees with other studies of cartilaginous airways in COPD (Tiddens, Pare et al. 1995), (Bosken, Wiggs et al. 1990). Even though the small airways are believed to be the major site of airways obstruction in COPD (Hogg.
Macklem et al. 1968), this study (Lofdahl et al.) reminds us that remodelling of the large airways is also evident. Studies of large numbers of both large and small airways should be performed to more clearly examine the contribution of airway remodelling and ECM alterations in COPD, and the effect that these changes may have on the pathogenesis of the disease.

1.12 Measuring the ASM: Stereology
No study on the ECM in asthma or COPD has estimated the volume fraction of the ECM within the ASM layer or the airway wall using stereological techniques in both large and small airways. Several studies have used area fractions based on tissue sections ranging from 3-5µm. However, the use of sections of this thickness means that matrix elements are likely to overlap with the ASM cells, making it difficult to estimate proportional changes in the ASM and ECM. Estimating the volume of ECM and other elements is problematic since the matrix has no defined borders or countable features (such as nuclei). Therefore, overlap (or over-projection) of various tissues on standard sections (~5µm) leads to overestimation of the volume fraction of ECM within the ASM layer (Howard and Reed 2005). To overcome this limitation the Delesse principle can be applied which is used effectively in geological sampling (Mouton 2002). Essentially in a solid, opaque object, the volume fraction of particles and “matrix” may be estimated from the area fraction of each particle seen on the cut, polished surface of that sample. In semi-opaque tissue samples however, the area fraction of objects or structures will tend to be over-estimated on anything but very thin sections due to overlap of the structures seen through the tissue thickness. The use of thin (0.5µm) airway sections eliminates the problem of tissue overlap without compromising normal airway structure.

Obtaining quantitative data is an essential step in studying the structural changes to the airway wall that occur in disease. However, there are various limitations of conventional morphometry. The size of the structure or object of interest, and its orientation and shape can be difficult to discern from a single random section of the lung. The problems encountered when quantifying airway dimensions can be overcome using stereology which follows an assumption-free set of protocols based on a mathematical foundation (Hsia, Hyde et al. 2010). These methods allow for the unbiased characterisation of irregular 3Dimensional (3D) objects from measurements made on a 2Dimensional (2D) section (Weibel, Hsia et al. 2007). In studies of normal and
remodelled airway (and other tissues) structure, the use of stereological techniques has now become the accepted standard.

Standard morphological measurements of the airways and lung have given rise to a number of problems which have prompted the use of stereology. Structures of interest in the lung are usually quite small and not easily accessible for study under the microscope. Structures may be located in different areas inside the lung (large airways vs parenchyma) or embedded within other structures in the lung (i.e. the ASM layer is located within the airway wall). This requires the lung to be sufficiently sectioned in order for the structure of interest (e.g. smooth muscle layer) or object of interest (e.g. ASM nuclei) to be accessible for examination. The problem is that when the lung is sectioned into thin enough slices for the object of interest to be examined, the 3D structure is then only visible on a 2D section and information about the objects size, shape and orientation are lost on the 2D profile. This can cause structures of very different shape to have the same or similar profile as the object of interest and therefore introduce errors (Figure 1.22) (Boyce, Dorph-Petersen et al. 2010). For example, a bundle of nerves in the airway wall can have a similar looking profile as the ASM layer if the ASM layer has been cut in cross-section. Additionally, it is not clear where in an object the section has been made. This is problematic when trying to relate observations back to the whole organ. For example, if an alveolus on a 2D section looks small then it may mean that the sectioned alveolus is small or it may be a large alveolus that has been sectioned close to its periphery (Weibel, Hsia et al. 2007). Without knowledge of the 3D structure, information obtained from a 2D profile has little meaning. The development of stereology techniques has helped to overcome these problems and allow for accurate morphological data to be obtained.

Figure 1.22: Sectioning a complex 3D structure results in loss of 3D information, as 3D structures are reduced to 2D profiles in the section plane. Dissimilar structures can produce similar profiles and vice versa (Boyce, Dorph-Petersen et al. 2010).
There are several advantages of stereological assessment of airway structures and dimensions. Stereology allows for quantitative estimation of an object of interest within a known volume. It does not rely on any assumptions of the shape, size orientation or distribution of the object of interest but rather uses a probe to sample the structure and relate it to the entire organ. An object in 3D space can be quantified by ‘throwing’ random geometric probes into the space and recording how they intersect with the object of interest (Boyce, Dorph-Petersen et al. 2010). These probes can include volumes, 2D sections, lines or points and the type of probe used is directly related to the feature being quantified (Figure 1.23). To select the correct probe the basic rule it to ensure that the dimensions of the feature being counted and the probe must sum to three. For example, when measuring ASM cells, the tip of the nucleus (a point, 0D) is counted when it first comes into focus and therefore requires a 3D probe. If measuring surface area (2D) of the basement membrane, a line probe (1D) must be used. Once a suitable geometric probe is selected, a feature can be quantified by counting the number of times it is intersected by the probe. By using this technique, unbiased estimates of volume, surface and length and number can be obtained.

<table>
<thead>
<tr>
<th>Object of interest</th>
<th>Probe</th>
<th>Counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Points – 0D</td>
<td>Volume – 3D</td>
<td>Number Density</td>
</tr>
<tr>
<td>Surface – 2D</td>
<td>Lines – 1D</td>
<td>Surface Density</td>
</tr>
<tr>
<td>Volume – 3D</td>
<td>Points – 0D</td>
<td>Volume Fraction</td>
</tr>
</tbody>
</table>

Figure 1.23: Relationship of the structural features and the appropriate corresponding probe. The first column illustrates the structural features that may be estimated by stereological methods: volume, surface area, and number. The second column illustrates the correct probe to sample the feature. The third row shows a visual representation of the probes interaction with the feature of interest. The dimensions of the object of interest and the probe must add up to three (Boyce, Dorph-Petersen et al. 2010).
Although the method of stereology is unbiased in its quantification of cell parameters, careful sampling techniques are required to ensure bias is not introduced at other stages. Measurements made using stereology are typically quantities per unit volume and as such need to be related back to the volume of the total organ of interest. This can be achieved through the use of unbiased, accurate and random sampling techniques to ensure that each part of the organ is sampled with equal probability. The most commonly employed method is to use the fractionator technique where the object of interest is split into a number of smaller pieces and a systematic, random sample of these are selected for use in subsequent stages (Figure 1.24) (Hsia, Hyde et al. 2010). This approach is continued until the sample is of workable size to allow the user to count the object of interest (e.g. smooth muscle cells). At the final sampling stage, the tissue section is isotropically oriented so that the object of interest within the tissue section has the same probability of being sampled, regardless of its orientation. However, this final step is not necessary when estimating ASM cell number as there are no special section orientation requirements to estimate volume (Boyce, Dorph-Petersen et al. 2010). Therefore, any section orientation can be used. With this technique the object of interest can then be related back to the whole organ by multiplying the count of the object of interest by the inverse sampling fraction which, in this example, would achieve an unbiased estimate of cell number.

![Fractionator sampling technique](image)

Figure 1.24: Fractionator sampling technique. The whole lung (A) is sectioned multiple times at a constant interval and a random start of the first cut. Sectioning of the object continues (B) until pieces are at a workable size (C). These pieces can then be embedded, sectioned and stained for stereological analysis (adapted from Hsia, Hyde et al. 2010).
The principals of stereology can be applied to the ASM layer and used to determine the presence of hypertrophy or hyperplasia. The number of ASM cells can be estimated by counting ASM nuclei using a 3D probe since the cells are mononuclear (Ebina, Takahashi et al. 1993), and the nuclei can easily be viewed as discrete, countable objects. This thesis examines the use of stereological techniques within the airway and applies them to the ASM layer in order to derive unbiased estimates of ASM size and volume in health and disease.

1.13 Aims & Organisation of Thesis

The area of the layer of ASM is increased in asthma and to a lesser extent in COPD, however remains unclear if hypertrophy and/or hyperplasia of airway ASM cells, and/or more extra-cellular matrix (ECM) contribute to this remodelling. In addition, there is debate surrounding the effects of the remodelling on airway function. Studies to quantify the ASM cell volume are few and none take into account the effects of the ECM when calculating ASM volume. Additionally, no studies have examined large numbers of subjects when assessing hypertrophy or hyperplasia, or determined the effects of multiple characteristics. Finally, the effects of the composition of the ASM on physiological behaviour of the airway, including airway narrowing and bronchodilatory response to DI, remain unclear.

Therefore, the specific aims of this project are to:

1. Refine methods for the stereological measurement of the composition of the ASM layer by examining the sources of variation that exist within and between airway segments.
2. Assess ASM cell hypertrophy and hyperplasia and volume fractions of ECM in the ASM layer normal subjects and in subjects with asthma and COPD.
3. Relate ASM and ECM parameters and dimensions of the airway wall (areas of smooth muscle, cartilage, total wall, mucous glands and epithelium; basement membrane thickness; volume fractions of elastin, collagen and other ECM proteins) and subject characteristics (age, sex, duration and severity of asthma, cigarette smoking and allergies) to the diagnosis of asthma.
4. Relate ASM and ECM parameters and subject characteristics (age, sex, cigarette smoking) to the diagnosis and severity of COPD.
5. Relate *in vitro* physiological properties of the isolated airway wall (airway narrowing and bronchodilatory response to DI) to ASM and ECM parameters and airway dimensions.

6. Determine whether the bronchodilator response to DI *in vivo* correlates with ASM and ECM parameters and airway dimensions.

In Chapter 1 a review of relevant literature is presented with a particular focus on what is known about the changes to the ASM layer in asthma and COPD. This thesis examines the composition of the ASM layer and determines the relative contribution of ASM hypertrophy, hyperplasia and ECM to the increased thickness of smooth muscle layer seen in these airway diseases. Chapter 2 details the general methods used to address the project aims including methods for stereological assessment of the ASM. Data and findings are presented in six result chapters (3-8).

Chapter 3 addresses the sources of variation that may affect the accuracy of stereological measurements of the ASM layer. Although stereology is now the accepted standard for measuring ASM parameters, little is known about the variability of the parameters to be measured, within and between airways. The aim of this study is to assess sources of variation in the measurement of the area of the ASM layer ($A_{ASM}$), the volume fraction of ASM cells ($V_{VASM}$) and the numerical density of ASM cells ($N_V$) within the ASM layer. The effect of sections thickness and airway size on estimates of these parameters will also be assessed. The results of this study informed subsequent experiments throughout this thesis. That is, we followed protocols determined to have the lowest variation in measurement.

Chapter 4 shows the results of using the above stereological techniques to examine the compositional changes to the ASM layer in asthma. The contribution of ASM hypertrophy or hyperplasia to the increased thickness of the ASM layer in asthma is examined. Importantly, possible changes to the ECM within the ASM layer are considered and measurements of ASM cell size and number are calculated after correcting for the amount of ECM. This study uses a large number of airway samples post-mortem from subjects who had died as a direct result of their asthma (fatal asthma), subjects with asthma who had died of non-respiratory causes (non-fatal asthma) and non-asthmatic subjects (controls).
Chapter 5 presents the results of functional studies of the airways from subjects with asthma and their relation to the structural remodelling of the airways. In asthma, functional abnormalities of the airway include exaggerated airway narrowing and a reduced bronchodilatory response to DI. Airway segments are studied in vitro. Fresh tissue (non-fixed) was acquired post-operatively. On the subsequently fixed tissue, airway dimensions, ASM cell size and number and the composition of the ASM layer (volume fractions of ASM, ECM and “other”) are measured. The relationship between the structure of the airways to airway narrowing, airway compliance and the pressure required to close the airways are examined.

Chapter 6 focuses on the composition of the ASM layer in COPD and how it differs from that present in asthma. The aim of this study is to examine whether the increased thickness of the smooth muscle in airways from COPD subjects is due to ASM hypertrophy or hyperplasia and how this relates to the severity of COPD. Few studies of this nature have been performed and previous similar studies have tended to focus on more severe cases of COPD. I compare my results with those found in asthma, to determine the relative contributions of the ECM and smooth muscle volumes to the ASM layer in these conditions.

Chapter 7 explores the relationship between cigarette smoking and the structure and functional responses of the ASM layer. In previous chapters, structural differences between asthma and COPD are identified which suggest that the underlying disease mechanisms differ. These structural abnormalities may explain apparent differences in the functional behaviour of subjects with asthma and COPD. In this chapter, the role of cigarette smoking on airway mechanical properties was assessed, with the view that the high prevalence of cigarette smoking in COPD contributes to the separation from asthma. Similarly to Chapter 5, airway segments are obtained following lung resection surgery and studied in vitro to assess airway function. On the subsequently fixed tissue, airway dimensions, ASM cell size and number and the composition of the ASM layer (volume fractions of ASM, ECM and “other”) are measured. The chapter relates cigarette smoking pack years, airway mechanical properties (e.g. compliance) and the structure of the ASM layer in human bronchi.

Chapter 8 related ASM and ECM parameters of the ASM layer to the in vivo response to DI. That is, unlike previous chapters where bronchial segments in vitro were used to
relate the isolated airway response to local structural properties, in this chapter the relationship with the whole lung response to DI will be examined. A modified lung function test, incorporating a partial and maximal expiratory manoeuvre, is performed on subjects prior to surgery to allow the response to DI to be measured (bronchodilation or bronchoconstriction). By this approach, the difference in flow after maximal expiration is compared with flow after a partial expiration without DI, and used to determine the effect of DI. Airway segments are collected following lung resection surgery and fixed to assess airway structure.

Each of the results chapters (3-8) includes a brief introduction outlining the aims, relevant background and experimental protocols, followed by a results section and discussion of the major findings. Chapter 9 is a general discussion of the major findings of this thesis, their contribution to current literature and final conclusions. All previously published data has been presented at the beginning of the thesis. Permissions from all co-authors for inclusion of published material in this thesis have been obtained and their contributions to the work in the thesis are acknowledged.
CHAPTER 2 - Methods

The investigations in this thesis comprise morphometric and stereological studies on fixed human lung tissue and physiological investigations using fresh tissue (non-fixed bronchi) in organ bath chambers. Tissues were obtained from both archival tissue banks (“Post-mortem tissue” - Chapters 3 and 4) and from subjects undergoing lung surgery to remove pulmonary neoplasms (“Post-operative tissue” Chapters 5 - 8).

Approval for use of post-mortem tissue was obtained from the institutional ethics committees of the participating centres and from the Sir Charles Gairdner Hospital Human Research Ethics Committee (N° 2005-004). Participating centres include the Victorian Institute to Forensic Pathology, Melbourne, Australia (N° 41/93); the University of British Columbia, Vancouver, Canada (N° H06-03284); the University of Calgary, Calgary, Canada (N° 1376); the University of Sao Paulo, Brazil (N° 439/05) and the Institute of Child Health, London, United Kingdom (N° 05/Q0508/44). Use of post-operative tissue was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee (N° 2007-161) and The Mount Hospital Ethics Committee (N° EC68.1). These ethics approvals are also recognised by the University of Western Australia Human Research Ethics Committee, without the requirement of separate ethical approvals.

2.1 Subject Classification

Subject groups studied included asthma, chronic obstructive pulmonary disease (COPD), or control. Two methods for classification of asthma were used depending on available patient data and the nature of the study. For the histological studies presented in Chapter 4, utilising tissue acquired post-mortem, the diagnosis of asthma was based on information obtained from next-of-kin, hospital files, coroners’ files, and subjects’ usual medical practitioners. Asthma subjects were further categorised as “non-fatal asthma” (non-respiratory cause of death, but with a history of asthma) and fatal asthma (died as a direct result of asthma). Clinical severity of asthma was defined using modified guidelines from the Global Initiative for Asthma (Masoli, Fabian et al. 2004). Subjects were categorised as having severe asthma if they had a history of ever using oral corticosteroids, ever admitted to hospital for asthma or daily symptoms. Subjects were classified as having moderate asthma if they had no oral corticosteroid use or
hospitalisations but had symptoms on most days or nights (more than three days per week), used regular inhaled corticosteroids or used reliever medications on most days or nights. Cases not having any of the above were categorised as mild asthma. For the physiological studies in Chapter 5 where airway tissues were acquired post-operatively, the diagnosis of asthma was based on a positive pre-operative response to the question, “Has your doctor ever told you that you have asthma?”.

Subjects were categorised as having COPD, and severity was determined, based on spirometry according to the Global initiative for Obstructive Lung Disease (GOLD) criteria (Table 2.1). We defined airflow obstruction as a post-bronchodilator FEV₁/FVC of <0.7. Mild, moderate, severe and very severe COPD were classified based on the FEV₁ (% predicted). Where fewer cases were available, the presence of obstructive lung disease was determined using FEV₁% predicted with obstructive disease having a post-bronchodilator FEV₁ <80% predicted. Control subjects had no reported history of asthma or other lung disease. Where available, lung function results (FEV₁/FVC >0.7 and/or FEV₁ >80% predicted) were also used to classify subjects as control.

Table 2.1: COPD disease severity classification using GOLD criteria when FEV₁/FVC ratio is <0.7 (Decramer et al. 2012).

<table>
<thead>
<tr>
<th>GOLD Stage</th>
<th>Severity</th>
<th>FEV₁ % Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mild</td>
<td>≥ 80</td>
</tr>
<tr>
<td>II</td>
<td>Moderate</td>
<td>50 – 80</td>
</tr>
<tr>
<td>III</td>
<td>Severe</td>
<td>30 – 50</td>
</tr>
<tr>
<td>IV</td>
<td>Very Severe</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

2.2 Tissue Origin and Use

Archival lung tissue banks had previously been established as part of post-mortem studies investigating histological changes in the airways and lungs in patients with asthma and COPD. Post-mortem tissue for this thesis were sourced from our own tissue bank in Perth (Australia), and through collaboration with other groups in Melbourne (Professor Michael Abramson), Sydney (Dr Karen McKay), Auckland and Vancouver (Professor Tony Bai), Calgary (Professor Francis Green) and São Paulo (Professor
Thais Mauad). These tissue banks were originally set up for a number of studies examining mechanisms of airway narrowing in asthma, normal airway structure and causes of asthma mortality. These studies included “Investigation of the epithelial reticular basement membrane in human airways” (McKay and Hogg 2002), “Prairie Provinces Asthma Study” (Green, Williams et al. 2010; Tough, Green et al. 1996), “Asthma management and mortality from Asthma” (Abramson, Bailey et al. 2001) and “Airway structural phenotypes in asthma” (Bai 1991). Collaboration between these research groups has allowed continued use of these tissues resulting in numerous publications.

Post-operative tissues were sourced in Perth from subjects undergoing lung tumour resection during the period of this project (2009-2012). I recruited and performed all associated lung function measurements on these subjects (see below). Archival post-operative tissues were also sourced from the laboratory of Professor James Hogg in Vancouver. Subjects in Vancouver had previously undergone a lung resection or lung transplant surgery. The origins of the tissue used in each of the studies described in Chapters 3 - 8 are specified below (Table 2.2 and 2.3).

Table 2.2: Post-mortem tissue use.

<table>
<thead>
<tr>
<th>Post-mortem study centre</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
<th>Chapter</th>
</tr>
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<tbody>
<tr>
<td>Perth</td>
<td>14</td>
<td>15</td>
<td>13*</td>
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<td>Auckland</td>
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<tr>
<td>São Paulo</td>
<td></td>
<td></td>
<td>5</td>
<td>4</td>
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</tbody>
</table>

City of origin, number of cases obtained, and tissue used in the different result chapters. Disease classification is indicated as control, non-fatal asthma or fatal asthma. * One case had no patient files (“unclassified”) but was categorised as fatal asthma by the large amount of ASM
Table 2.3: Post-operative tissue use.

<table>
<thead>
<tr>
<th>Post-operative tissue source</th>
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<th>Asthma</th>
<th>COPD</th>
<th>Chapter</th>
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<tbody>
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<td>Perth</td>
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<td>7</td>
<td>9</td>
<td>5 - 8</td>
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<tr>
<td>Vancouver</td>
<td></td>
<td></td>
<td></td>
<td>5 - 8</td>
</tr>
</tbody>
</table>

City of origin, number of cases obtained, and tissue used in the different result chapters. Disease classification is indicated as control, asthma or COPD.

2.3 Patient Recruitment

I recruited subjects undergoing lung surgery at Perth hospitals; Sir Charles Gairdner Hospital and The Mount Hospital, and obtained patient approval to use post-operative tissue in our histological and physiological studies. Once ethics approval was obtained, the study was set up by contacting the cardiothoracic surgeons and obtaining permission from them to approach their patients. Discussions with pathology staff from Sir Charles Gairdner Hospital and The Mount Hospital were undertaken to provide information on the study and explain what would be involved. Presentations were also made to other pathology staff to introduce myself and give further details on the project.

Potential patients for inclusion in the study at Sir Charles Gairdner Hospital were identified on a weekly basis from the database of patients scheduled for preadmission clinics. Patients scheduled to undergo lobectomy or pneumonectomy were identified and contacted by phone to discuss participation in the study. Patients from The Mount Hospital due to undergo lung resection and who were potentially suitable for participation, were identified by their cardiothoracic surgeon Mr Mark Edwards. These patients were also contacted by phone for inclusion in the study. Subjects willing to be involved were met prior to surgery either at the preadmission clinic (Sir Charles Gairdner Hospital) or in the ward on the day prior to surgery (The Mount Hospital). The patients were informed of the study, given an opportunity to ask questions about the study and if satisfied, provided written consent. Participation rate in the study was >95% of subjects contacted. Subjects who did not wish to participate in the study stated lack of time and high levels of current stress as reasons for declining involvement.
2.3.1 Questionnaire and Allergy Tests

Patients who provided consent completed a questionnaire on respiratory symptoms, smoking history, previous medical history and current medication use (Appendix 3). Smoking history was used to categorize subjects as never-smokers, current smokers or ex-smokers (have not smoked in 1 year or more). Cigarette smoke exposure was quantified as pack-years (Section 2.8.4).

Subjects underwent skin prick (anterior forearm) response testing to a range of common household allergens. The allergens tested were as follows: house dust mite (Dermatophagoides pteronyssinus), mould (Alternaria tenuis and mould mix: Alternaria tenuis, Aspergillus, Fusarium vasinfectum, Helminthosporium interseminatum, Hormodendrum cladosporioides, Mucor racemosus, Penicillium, Phoma herbarum, Pullularia pullulans, Rhizopus nigricans), grass (grass mix: Kentucky Bluegrass, Orchard, Redtop, Timothy, Sweet Vernalgrass, Meadow Fescue, Perennial Ryegrass), cat dander and dog hair. Histamine and saline were used as positive and negative controls, respectively. The test was performed in accordance with the Australasian Society of Clinical Immunology and Allergy Guidelines (ASCIA 2006), and is detailed in Appendix 4. Allergy testing was performed on the left forearm by administering a drop of allergen to the surface of the skin and scratching the skin with sterile lancets. A separate lancet was used for each allergen tested. The allergen was removed after one minute and the level of allergic response determined 15 minutes following allergen exposure. The size of the wheal formed was measured and a positive allergic response (atopy) defined as a wheal diameter of 3mm or greater.

2.3.2 Spirometry – Maximal and Partial Respiratory Manoeuvres

Spirometry was performed using a wedge spirometer (model 570, Med Science) according to ATS criteria (Miller 2005), both before and 15 minutes after inhalation of three puffs of salbutamol (100μg/puff) from a metered dose inhaler. During the test the performance of each subject was visually assessed and the spirometry readout checked for quality. Satisfactory manoeuvres required a maximal inspiration to total lung capacity, a fast exhalation of air with minimal hesitation and full expiration to residual volume. Expiration continued for more than six second to ensure end of test criteria were met. Cough or obstruction of the mouthpiece resulted in exclusion of that test.
To determine the response to deep inspiration (DI), a wedge spirometer was used. The wedge spirometer uses a leak free, closed airflow system which allows multiple manoeuvres to be performed in succession. As it is a closed system, the spirometer was flushed between breathing manoeuvres to avoid rebreathing and carbon dioxide accumulation. The expertise and maintenance of this system was provided by Mr William Noffsinger from the Department of Pulmonary Physiology and Sleep Medicine at Sir Charles Gairdner Hospital.

For the breathing manoeuvres using the wedge spirometer (Figure 2.1) subjects were required to breathe normally (tidal breathing) for a few breaths before taking a partial inspiration (to end inspiratory tidal volume) and exhaling forcefully to residual volume. This was immediately followed (without the subject taking his/her mouth off the end of the mouthpiece) by a maximal inspiration to total lung capacity and a maximal forced expiratory manoeuvre to residual volume. This procedure was repeated until three reproducible manoeuvres were obtained, where the difference in FEV\(_1\) was <200ml between breaths. Each manoeuvre was separated by a period of three minutes to allow for the reversal of the effects of a deep inspiration on airway motor tone (King, Moore et al. 1999). From the three reproducible maximum expiratory efforts, the resulting time data were used to determine the best FEV\(_1\) and FVC as discussed in the introduction. The response to DI was calculated at 60% of the vital capacity (0.6 FVC) as a ratio of the flow measured during maximal (M) expiration to that measured during partial (P) expiration (i.e. the M/P ratio) (Berry and Fairshter 1985, Pellegrino, Confessore et al. 1999).
Figure 2.1: Example trace of the breathing manoeuvre performed by subjects to assess response to DI. Vertical dashed line indicates approximately 0.6 FVC. The M/P ratio of <1 indicates bronchoconstriction to DI in this subject. Volumes are relative not absolute.

2.4 Tissue Fixation and Processing

Post-mortem lungs were fixed by inflation with formalin at 25cmH₂O. Post-operative lungs were fixed by formalin immersion. Central (lobar) airways to the sub-segmental level were dissected and cut in cross section. Blocks of lung parenchyma containing small airways were cut from random locations from within the lobe. The number and location of airways obtained per case varied depending on tissue availability. Airway and lung tissue were embedded in paraffin. All airway tissues were cut in transverse orientation so that entire airway cross-section could be studied. An average of three large and three small airways were studied per subject in control subjects and in cases of non-fatal asthma, fatal asthma and COPD.
Airways obtained post-operatively from Perth hospitals were initially studied in organ bath chambers (Section 2.9), and were subsequently fixed in 4% formaldehyde at zero cmH₂O. The fixed airways (2 - 4cm in length) were cut into three segments which were processed and embedded in paraffin wax.

Transverse sections of 0.5µm, 4µm or 30µm thickness were cut and stained as required. In order to cut thin sections, the wax blocks were kept cold by sitting them in a container of dry ice before sectioning. The section thickness required and staining protocols used varied depending on the aim of the study (see Morphometry and Stereology below). Each staining procedure followed specific protocols which are detailed in Appendix 5. Histological stains used in this thesis were Mason’s Trichrome, haematoxylin and haematoxylin and eosin.

2.5 Airway Wall Morphometry

Planimetry was used to measure the perimeter of the basement membrane (Pbm), the areas of the ASM, inner wall, outer wall, total wall, cartilage and glands. The outer wall area was calculated from the area enclosed by the outer perimeter (Aₒ) minus the area enclosed by the outer perimeter of the ASM (Aₘₒ) (Figure 2.2). The inner wall area was calculated from Aₘₒ minus the area enclosed by the basement membrane (Abm) while the total wall area was the sum of inner and outer wall areas. Pbm was used as a marker of airway size.
Figure 2.2: Asthmatic airway depicting airway parameters measured. Perimeter of the basement membrane (Pbm – green), outer perimeter of the muscle (Pmo - balck), outer perimeter of airways (Pw), outer wall area (Aw - yellow), area of ASM (red), gland area (blue) and cartilage area (orange).

2.6 Estimating Components of the ASM Layer (ASM, ECM and Other)

Volume fractions ($V_V$) of ASM, ECM and “other” were estimated using the point counting technique at magnification x1000 on 0.5μm sections stained with Masson’s Trichrome. “Other” includes space due to fixation artefact, inflammatory cells and blood vessels. Point counting is a sampling technique that involves estimating the area of a feature of interest by counting the number of squares in a regular grid that lie within that feature (Figure 2.3). In practice, a grid overlay is applied to a micrograph and the numbers of points (intersections of the grid) that fall on the object of interest are counted. As each point represents a unit of area, the area of the object of interest can then be calculated (Section 2.8).
Figure 2.3: Point counting technique. The intersections of the squares (red) are counted within the object of interest (blue) and can be used to estimate the area of the object.

Using point counting, the ASM layer was systematically and randomly sampled around the airway circumference (Figure 2.4) at 12, 3, 6 and 9 o’clock positions and again at 1, 4, 7 and 10 o’clock if necessary until over 200 points falling on the areas of interest (ASM, ECM or ‘other’ - Figure 2.5) were counted. If no muscle was present at the specified o’clock position, the position was skipped and the next position in the sequence was sampled. Points were allocated to ASM (red), ECM (blue) and other (white) by their stain colour. Masson’s Trichrome stains muscle red, collagen and bone blue or green, the cytoplasm light red or pink and cell nuclei dark brown to black.

Figure 2.4: Thin (0.5μm) section used for point counting. The ASM layer (dashed outline) was sampled at x1000 magnification at 12, 3, 6, and 9 o’clock (boxes).
Figure 2.5: Magnified section of the ASM layer with point counting grid overlayed. The point counting technique was used to count ASM (red), ECM (blue) and other (white) within the ASM layer. Counting continues until over 200 points falling on ASM were counted.

2.7 Airway Smooth Muscle Stereology

2.7.1 Area of the ASM layer ($A_{ASM}$)
The total area of the ASM layer on each section was measured by delineating the outline of the ASM bundles around the airway wall (x40).

2.7.2 Numerical Volume Density ($N_V$) of ASM Cells
$N_V$ was estimated in randomly selected high-power fields (HPF, x1000 magnification) on 30µm sections of the airway wall (haematoxylin) using the optical dissector approach (Howard and Reed 2005) outlined below. Using stereological software (CASTGrid) a set of randomly selected fields was generated to count smooth muscle cells (Fig 2.6). At each HPF ASM cell numerical density was measured as follows. The depth of the tissue section was measured at ten points around the airway and the average used to determine the depth of the focal plane. Using the measured tissue thickness a “guard depth”, or margin, of approximately 5µm (within which no nuclei were counted) was added at the top and bottom of the tissue section to avoid irregularities at the cut surfaces. Nuclei were counted only when the dissector frame (the box within which nuclei can be
counted) was ‘active’ (turned from red to green) once the guard depth had been passed through (Figure 2.7). The volume of the optical disector was calculated as the area of the counting frame multiplied by the measured ‘active’ depth scanned through the tissue. ASM cell nuclei were counted when they first come into focus and only if they lay wholly or partly within the frame without crossing the exclusion (red) lines at any stage during sampling. For each airway the total number of cells counted (N) was divided by the total measured volume (V) of the disectors to obtain the ASM cell numerical density (N/V). The measured volume used is the depth of tissue (section thickness) in which the cells have been counted. Since the airway is cut in transverse section, the scanning direction through the depth of the disector is “along” the length of the airway, thus making it possible to express cells as a number per unit length of airway.

Figure 2.6: Transverse 30μm section of airway with the basement membrane (blue) and smooth muscle layer (green) delineated. Randomly generated fields within the smooth muscle layer (crosses) are magnified to count smooth muscle nuclei.
Figure 2.7: View of ASM layer (green indicates outline of ASM layer) and optical disector frame (box) at different depths within a tissue section. The optical disector frame is red within the guard areas at the top (5µm) and bottom (30µm) of the tissue section. No cells were counted when the frame was red. When the optical disector frame turns green, ASM nuclei may be counted according to specific criteria (see text). Nuclei which meet the counting criteria (ticks) and those which do not (crosses) are indicated above (10 - 25µm).

2.8 Calculations

Once point counting was performed and \( A_{\text{ASM}} \) and \( N_V \) (cells/mm\(^3\)) determined, the volume fraction of ASM, the average ASM cell volume and the number of ASM cells per airway length are estimated (Figure 2.8).
Figure 2.8: Diagram of a length of airway depicting thick and thin transverse sections and the calculations that can be derived from each.

2.8.1 Volume Fraction of ASM ($V_{VASM}$)

The volume fraction of actual smooth muscle ($V_{VASM}$) excluding other elements such as ECM was calculated using the point counting technique on thin (0.5μm) sections. The volume fraction of ASM ($V_{VASM}$) is given by the total number of points falling on ASM, divided by the sum of the points falling on ASM plus ECM plus other within the smooth muscle layer.

$$V_{VASM} = \frac{\Sigma \text{(points on ASM)}}{\Sigma \text{(points on ASM + ECM + other)}}.$$
2.8.2 Average Cell Volume ($V_C$)
ASM cell volume ($V_C$) was calculated as the inverse of the numerical density. Since $V$ includes all components of the ASM layer, it needs to be multiplied by the volume fraction of actual smooth muscle within the ASM layer ($V_{VASM}$) to avoid overestimation of average ASM cell volume.

$V_C$ (cells/$\mu$m$^3$) = $1 / (N_V \times V_{VASM})$

2.8.3 ASM Cell Number per unit length ($N_L$)
The average number of ASM cells per unit length of airway was calculated by multiplying $N_V$ by the area of ASM in which $N_V$ was determined and multiplying this by the airway length (usually 1mm).

$N_L$ (cells/mm) = $N_V \times L \times A_{ASM}$

2.8.4 Pack Years of Smoking
The number of cigarettes smoked per year (pack years) was determined using smoking information obtained from the respiratory questionnaire. The calculation for pack years is stated below, assuming 20 cigarettes per packet.

Pack years = ($N^o$ cigarettes smoked per day / 20) x total $N^o$ of years smoked

2.9 Organ Bath Experiments on Isolated Bronchial Segments
One or more segment of bronchi (>2cm length) from the lobar bronchus or other large identifiable airways were dissected post-operatively by pathology staff. The dissected airways were macroscopically normal and located either proximal to the site of the peripheral tumour or in another bronchial segment not required for pathological staging. Fresh tissue segments were prepared for in vitro measurements of airway function by co-investigators (Drs Peter B. Noble and Peter K. McFawn) from the School of Anatomy, Physiology and Human Biology at UWA.

The airways were dissected free from surrounding lung parenchyma and all side branches ligated to produce a leak-free airway tube. Airway segments were cannulated at each end and mounted horizontally in an organ bath chamber (Figure 2.9) containing
gassed (95%O$_2$/5%CO$_2$) Krebs solution (mM: 121 NaCl; 5.4 KCl; 1.2 MgSO$_4$; 25 NaHCO$_3$; 5.0 sodium morpholinopropane sulfonic acid; 11.5 glucose; and 2.5 CaCl$_2$) warmed to 37°C. Segments were stretched to a length approximating that present at functional residual capacity. Transmural pressure was initially set at 5cmH$_2$O and varied to simulate normal breathing and intermittent DI’s. All recordings were made under pressure-controlled conditions where the transmural pressure was controlled and narrowing of the segment measured.

![Airway segment](image)

Airway segment

Airway branch tied off

Cannulated ends

Figure 2.9: Human airway segment collected post-operatively and mounted in an organ bath chamber.

Airway narrowing to bronchoconstriction, bronchodilation to simulated DI, airway compliance and the pressure required to fully collapse the airway were measured on each airway. This was achieved using a motorised syringe pump (Figure 2.10) comprising a glass syringe (1ml glass syringe Mod 1001, Hamilton Company, Nevada, USA) driven by a feedback controlled servo motor (M540, McLennan Servo Supplies Ltd., Surrey, UK) and motor controller which maintained the lumen pressure at the desired static or oscillating value (see below). All driving software was written in C programming language (Shane De Catania, Western Australia). Changes in airway luminal volume (e.g. airway narrowing, inflation or deflation) were measured by a displacement transducer (HEDS-5540#A06, RS Components, Western Australia) that measured the rotation of the syringe motor. Volume changes were calibrated to graduations on the syringe. A pressure transducer (MLT0380/D, ADInstruments, New South Wales, Australia) connected to a luminal port measured intraluminal pressure and therefore transmural pressure. The motor controller constantly monitored transmural pressure through a feedback loop which added or removed volume to the lumen using
the syringe to maintain the set pressure. Airway narrowing (decrease in airway volume) was determined from the volume change required to keep pressure at the set point. Volume and pressure signals were recorded by a PowerLab data acquisition system (ADInstruments, New South Wales, Australia).

Figure 2.10: Schematic of the syringe pump oscillator and organ bath system

2.10 Organ Bath Experimental Protocol

After dissection, airways were allowed to equilibrate to organ bath conditions for approximately one hour at a transmural pressure of 5cmH$_2$O. Tissue viability was then confirmed using electrical field stimulation via platinum electrodes and then by $10^{-4}$M acetylcholine (ACh), followed by 30 minute of wash out and recovery. Cumulative dose-response curves to ACh ($3\times10^{-6}$M to $3\times10^{-3}$M) were performed under static conditions with transmural pressure held fixed at 5cmH$_2$O, and under dynamic conditions simulating tidal oscillations with intermittent DI (the order of protocols was randomised). Tidal breathing comprised transmural pressure oscillations from 5-10cmH$_2$O at 0.25Hz and each DI comprised a two second linear ramp up from 5-30cmH$_2$O, a hold at peak pressure for two seconds, and a two second linear ramp down to 5cmH$_2$O (LaPrad, West et al. 2008). For the dynamic protocol DI was applied after airway narrowing at each concentration of ACh had stabilised. Re-narrowing after DI was monitored until one minute before the next dose of ACh was administered. Prior to each dose-response curve airways were preconditioned for 12 minutes: static with transmural pressure fixed at 5cmH$_2$O; dynamic with tidal oscillation and DIs every six minutes to simulant the rate of spontaneous sighs in human subjects (Bendixen, Smith et al. 1964).
A second protocol was used in a subset of airways (Chapter 5) to determine the relationship between the amplitude (pressure) of DI and the bronchodilator response. Tidally oscillating airways were narrowed to carbachol with dose modified to match the level of narrowing between groups (approximately 25 - 30% decrease in airway volume). Once airway narrowing to carbachol had plateaued each of three different sized DIs were administered in randomised order with the peak pressures equating to 15, 30 and 60cmH₂O transmural pressure. The subsequent DI was administered once re-constriction to the previous DI had stabilised.

Once the above protocols were complete, ASM tone was allowed to reverse (regular bath washing with Krebs solution for up to two hours after carbachol), and theophylline (10⁻²M) was added to the bath to identify intrinsic ASM tone. At the end of the experiment the volume of the relaxed airway at 5cmH₂O was determined from the volume that could be withdrawn until closure/collapse. Finally, pressure (cmH₂O) required to collapse the airway was recorded.

Following in vitro studies the airway segments were fixed in formalin for measurements of airway wall dimensions (as described above and in Section 2.5).

2.11 Statistical Analysis

Statistical analyses were performed using SigmaPlot 12 or Prism. Specific analyses are detailed in each chapter. Generally, all data analysis was checked for normality using the Shapiro-Wilk test with an equal variance test performed if data were normally distributed. Non-parametric post-hoc tests of significance were used as appropriate. Throughout this thesis, the accepted level of significant is p<0.05. Parameters from subjects with multiple airways were assessed using subject means. Airways were categorised as small, medium or large using Pbm to determine airway size. Multiple linear regression models were used in Chapter 4 where large numbers of airways from a number of subjects and sites were analysed.
CHAPTER 3 - Estimating Airway Smooth Muscle Cell Volume and Number in Airway Sections: Sources of Variability

3.1 Introduction

Increased thickness of the ASM layer has been identified in airway diseases including asthma and COPD and alterations in the mechanical behaviour of this layer are likely to play a role in abnormal airway function. The ASM layer in a segment of airway can be thought of as a sleeve within the airway wall. This layer is composed of ASM cells, 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) (Heard and Hossain 1973; Woodruff, Dolganov et al. 2004), increased size of ASM cells (hypertrophy) (Ebina, Yaegashi et al. 1990; Regamey, Ochs et al. 2008), an increase in the volume of other elements such as the ECM, or combinations of all of these changes. Since different pathological processes may contribute to hypertrophy or hyperplasia of ASM cells in airway diseases (Bentley and Hershenson 2008), estimation of the size and number of ASM cells and the volume fraction of ECM in intact airways will give insight into possible disease mechanisms. However, currently little is known about the sources of variation in measurements undertaken to estimate these parameters.

The number and size of ASM cells within the ASM layer can be estimated knowing the volume of the ASM layer per unit length of airway, the volume density of ASM cells \((N_V)\) and the volume fraction of smooth muscle \((V_{VASM})\), which excludes other elements such as ECM, within the ASM layer. The volume of the ASM layer in a length of airway is calculated from the area of the ASM layer seen on a sampled transverse section \((A_{ASM})\) multiplied by length. \(N_V\) may be estimated by sampling a known volume of the ASM layer using the optical dissector approach (Sterio 1984; James, Elliot et al. 2012) on ‘thick’ sections (30μm). This accommodates efficiency of sampling and ensures sufficient counting events (ASM cell nuclei). As we require only the volume of the smooth muscle, \(V_{VASM}\) must be calculated to avoid over-estimating 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.
(Howard and Reed 2005). To overcome this problem, “thin” sections must be used, although the optimal practical thickness is unknown.

Stereology has become the common technique for measuring ASM parameters in the assessment of hypertrophy and hyperplasia in airway disease. However, little is known about the variability of these parameters within and between airways. ASM parameters may vary within airway sections, along the length of airway segments and between airways of different sizes. The aim of the present study was to assess sources of variation in the measurement of $A_{ASM}$, $V_{VASM}$ and $N_V$ within the ASM layer. In large and small airways we examined, variation of tissue components between different transverse sections along an airway segment, the effect of the number and location of sampling sites with transverse sections on estimates of $N_V$ and the effect of tissue section thickness on estimates of $V_{VASM}$.

3.2 Methods

Although stereology has become the accepted technique for measuring the ASM layer, the sources of variation that may exist in these techniques are unknown. As such, we have used a series of experiments to assess sources of variation that exist in the measurement of the above parameters ($A_{ASM}$, $V_{VASM}$ and $N_V$) both within and between tissue segments. We examined four main sources of variation to determine:

1. The variation in $A_{ASM}$ and $V_{VASM}$ with different section thicknesses (0.5μm, 4μm or 30μm).
2. The effect of the number of sampling regions and the site on estimates of $N_V$.
3. How measurements of $A_{ASM}$, $V_{VASM}$ and $N_V$ vary along the length of the airway.
4. The effects of airway size on estimates of $A_{ASM}$, $V_{VASM}$ and $N_V$.

3.2.1 Subjects

Post-mortem tissues from Perth, Australia from both control and asthmatic subjects were used for this study.

3.2.2 Area of the ASM layer ($A_{ASM}$)

The effect of section thickness on the estimated area of the ASM layer was assessed in a large airway (Pbm>10mm) from each of nine subjects on consecutive 0.5μm, 4μm or
sections, stained with haematoxylin and eosin. Variability of $A_{ASM}$ along the length of airway segments was assessed in one large and one small airway each from three subjects (control, asthma, unclassified). From a random starting point, a 1mm length of airway was sectioned (0.5$\mu$m for up to 10$\mu$m plus three 30$\mu$m sections) every 100$\mu$m until tissue exhaustion. The effects of airway size (Pbm 2 – 40mm) on $A_{ASM}$ were compared in at least five airways from each of 12 subjects.

### 3.2.3 Volume Fraction of ASM ($V_{VASM}$)

The effect of section thickness on estimated $V_{VASM}$ were assessed in one large and one small airway using tissue sections of 0.5$\mu$m, 4$\mu$m and 30$\mu$m thickness each from two subjects using the point counting technique. On three separate counts on each section, the ASM layer was systematically and randomly sampled around each transverse section until $>$200 points falling on either ASM or ECM or “other” were counted. For example, for the separate 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 were present, the sum of the separately estimated fractions should equal one. Variability of $V_{VASM}$ along airway segments and the effects of airway size were also assessed, as for $A_{ASM}$.

### 3.2.4 Numerical Volume Density ($N_{V}$) of ASM Cells

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 high-power fields had been sampled. The effect of sampling site around airway 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 when the whole smooth muscle layer was sampled. The variability of $N_{V}$ along airway segments and the effects of airway size were also assessed, as for $A_{ASM}$.

### 3.3 Results

#### 3.3.1 Subjects

The number of subjects, subject demographics and average airway size for each group are presented in Table 3.1.
Table 3.1: Subject characteristics.

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Gender M/F</th>
<th>Age years</th>
<th>Weight kg</th>
<th>Height cm</th>
<th>Smoking Y/N/Ex/U</th>
<th>Pbm mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 12)</td>
<td>11 / 1</td>
<td>27 ± 11</td>
<td>79 ± 20</td>
<td>172 ± 7</td>
<td>2 / 2 / 1 / 7</td>
<td>10.7 ± 8.4</td>
</tr>
<tr>
<td>Non-fatal asthma (n = 8)</td>
<td>2 / 6</td>
<td>30 ± 10</td>
<td>74 ± 33</td>
<td>159 ± 11</td>
<td>4 / 1 / 0 / 3</td>
<td>11.4 ± 10.0</td>
</tr>
<tr>
<td>Fatal asthma (n = 11)</td>
<td>7 / 4</td>
<td>49 ± 20</td>
<td>65 ± 16</td>
<td>171 ± 11</td>
<td>2 / 2 / 4 / 3</td>
<td>8.8 ± 7.1</td>
</tr>
<tr>
<td>Unclassified (n = 1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.1 ± 6.9</td>
</tr>
</tbody>
</table>

Not all subjects were used for all analyses. Abbreviations: Ex = Ex smokers; F = Female; M = Male; N = Non-smokers; Pbm = Perimeter of basement membrane; U = Unknown smoking status; Y = Current smokers

3.3.2 Area of the ASM layer ($A_{ASM}$)

3.3.2.1 Effect of Tissue Thickness

Compared with 0.5μm sections, the estimated $A_{ASM}$ was increased by an average of 1.6% and 12.1% in 4μm and 30μm sections respectively in control subjects and by 12.2% and 28.8% in 4μm and 30μm sections respectively in fatal asthma subjects (Figure 3.1). The mean differences in measured $A_{ASM}$ between 0.5 and 30μm sections of the same large airways were 0.13mm² for control subjects and 0.29mm² for fatal asthma cases.
Figure 3.1: Measured cross-sectional area of airway smooth muscle (A\textsubscript{ASM}) in 0.5\textmu m, 4\textmu m and 30\textmu m sections of large airways from five control subjects and four cases of fatal asthma.

3.3.2.2 Effect of Different Sections Along a Length of Airway

When the variation in A\textsubscript{ASM} was examined on 30\textmu m sections along a 1mm length of airway, the coefficient of variation (CV) ranged from 9.1 – 27.7\% and was not related to the absolute value (Table 3.2). The mean differences in A\textsubscript{ASM} in 30\textmu m sections between the control, asthma and unclassified subjects were 0.12mm\textsuperscript{2}, 0.10mm\textsuperscript{2} and 0.51mm\textsuperscript{2}. These equate to differences of 13\%, 9\% and 14\% for the mean absolute areas, respectively.
Table 3.2: Variability of dimensions and components of the airway smooth muscle layer in transverse sections along 1mm lengths of small and large airways.

<table>
<thead>
<tr>
<th></th>
<th>Small Airways (n=26 sections)</th>
<th></th>
<th>Large Airways (n=27 sections)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measured Values (mean ± SD)</strong></td>
<td></td>
<td><strong>CV, %</strong></td>
<td></td>
</tr>
<tr>
<td>Subject</td>
<td>Pbm (mm)</td>
<td>$A_{ASM}$ (mm$^2$)</td>
<td>$N_V$ cells/μm$^3$ x10$^{-4}$</td>
</tr>
<tr>
<td>C</td>
<td>3.6 ± 0.4</td>
<td>0.03 ± 0.01$^\ddagger$</td>
<td>1.91 ± 0.37$^\ddagger$</td>
</tr>
<tr>
<td>FA</td>
<td>5.0 ± 0.3</td>
<td>0.10 ± 0.02$^\ddagger$</td>
<td>1.72 ± 0.27</td>
</tr>
<tr>
<td>U</td>
<td>4.1 ± 0.4</td>
<td>0.10 ± 0.02$^\ddagger$</td>
<td>1.79 ± 0.27$^\ddagger$</td>
</tr>
<tr>
<td>CV, %</td>
<td>11.2</td>
<td>25.6</td>
<td>19.4</td>
</tr>
<tr>
<td>FA</td>
<td>6.4</td>
<td>18.5</td>
<td>15.6</td>
</tr>
<tr>
<td>U</td>
<td>10.7</td>
<td>15.5</td>
<td>15.3</td>
</tr>
</tbody>
</table>

| Subject | Pbm (mm) | $A_{ASM}$ (mm$^2$) | $N_V$ cells/μm$^3$ x10$^{-4}$ | $V_{VASM}$ |
| C       | 16.6 ± 0.9 | 0.90 ± 0.17 | 1.45 ± 0.39 | 0.69 ± 0.06 |
| FA      | 12.2 ± 0.6 | 1.15 ± 0.10 | 1.74 ± 0.26 | 0.69 ± 0.05 |
| U       | 17.5 ± 1.0 | 2.00 ± 0.55$^\ddagger$ | 1.40 ± 0.32 | 0.67 ± 0.04 |
| CV, %   | 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 |

Abbreviations: $A_{ASM}$ = Area of ASM; 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 <0.02 vs. C; † p <0.02 vs. FA; ‡ p = <0.05 vs. large airways.

3.3.2.3 Effects of Airway Size

$A_{ASM}$ increased significantly with airway size in all subject groups (Table 3.3).
Table 3.3: Effect of airway size on airway smooth muscle area, density of airway smooth muscle cells and volume fraction of ASM in control (n = 4), non-fatal asthma (n = 4) and fatal asthma (n = 4) subjects.

<table>
<thead>
<tr>
<th>Case</th>
<th>Pbm mm</th>
<th>(A_{ASM}) mm(^2)</th>
<th>(N_V) cells/(\mu m^3) x10(^4)</th>
<th>(V_{VASM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3.5 ± 1.3</td>
<td>0.09 ± 0.05</td>
<td>1.84 ± 0.32</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>NFA</td>
<td>3.1 ± 0.6</td>
<td>0.08 ± 0.03</td>
<td>2.08 ± 0.36</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>FA</td>
<td>2.9 ± 1.1</td>
<td>0.11 ± 0.10</td>
<td>1.92 ± 0.53</td>
<td>0.69 ± 0.07</td>
</tr>
<tr>
<td>All</td>
<td>3.2 ± 1.0</td>
<td>0.09 ± 0.06</td>
<td>1.95 ± 0.31</td>
<td>0.68 ± 0.07</td>
</tr>
</tbody>
</table>

Medium, n = 23 airways

<table>
<thead>
<tr>
<th>Case</th>
<th>Pbm mm</th>
<th>(A_{ASM}) mm(^2)</th>
<th>(N_V) cells/(\mu m^3) x10(^4)</th>
<th>(V_{VASM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>6.7 ± 1.0</td>
<td>0.23 ± 0.11</td>
<td>1.46 ± 0.20</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>NFA</td>
<td>7.1 ± 1.6</td>
<td>0.36 ± 0.18*</td>
<td>1.79 ± 0.46</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>FA</td>
<td>6.3 ± 1.6</td>
<td>0.53 ± 0.37</td>
<td>2.11 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>6.7 ± 1.4</td>
<td>0.38 ± 0.27</td>
<td>1.79 ± 0.48</td>
<td>0.65 ± 0.06</td>
</tr>
</tbody>
</table>

Large, n = 24 airways

<table>
<thead>
<tr>
<th>Case</th>
<th>Pbm mm</th>
<th>(A_{ASM}) mm(^2)</th>
<th>(N_V) cells/(\mu m^3) x10(^4)</th>
<th>(V_{VASM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>19.1 ± 11.0</td>
<td>1.24 ± 0.86*†</td>
<td>1.70 ± 0.39</td>
<td>0.57 ± 0.09*</td>
</tr>
<tr>
<td>NFA</td>
<td>20.7 ± 9.8</td>
<td>0.98 ± 0.94*</td>
<td>1.69 ± 0.29</td>
<td>0.66 ± 0.11</td>
</tr>
<tr>
<td>FA</td>
<td>20.2 ± 10.1</td>
<td>2.02 ± 1.37*</td>
<td>2.09 ± 0.90</td>
<td>0.65 ± 0.16</td>
</tr>
<tr>
<td>All</td>
<td>20.0 ± 9.9</td>
<td>1.29 ± 1.04</td>
<td>1.78 ± 0.48</td>
<td>0.62 ± 0.12</td>
</tr>
</tbody>
</table>

Abbreviations: \(A_{ASM}\) = Area of ASM; C = control; FA = fatal asthma; NFA = non-fatal asthma; \(N_V\) = ASM cell numerical density; Pbm = Perimeter of basement membrane; \(V_{VASM}\) = Volume fraction of ASM cells; Small airways = Pbm<5mm; Medium airways = Pbm 5 - 10mm; Large airways = Pbm >10mm. * = p <0.05 vs. small airways; † p = 0.05 vs. medium airways.

3.3.3 Numerical Volume Density (\(N_V\))

3.3.3.1 Number and Site of Samples

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 3.2). This was independent of the sampling site around the airway circumference.
Figure 3.2: Running means of airway smooth muscle cell numerical density ($N_V$) for five airways

3.3.3.2 Variation Along a Length of Airway

The variability of $N_V$ between sections taken 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 3.2). The between-airway (from the same person) CV for control subjects was 16.3% in the small airways and 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 3.2). The same patterns were seen in subjects with fatal asthma.

3.3.3.3 Effects of Airway Size

The absolute values of $N_V$ were not significantly different between airway sizes (Table 3.3).
3.3.4 Volume Fraction of ASM and ECM

3.3.4.1 Effect of Tissue Thickness

The separately estimated fractions of $V_{VASM}$ and $V_{VECM}$ increased with section thickness while $V_{VOther}$ (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 0.5µm sections to 1.10 and 1.19 in 4µm sections and 1.26 and 1.23 in 30µm sections (Table 3.4).

Table 3.4: The volume fractions of ASM components.

<table>
<thead>
<tr>
<th></th>
<th>0.5µm C</th>
<th>0.5µm FA</th>
<th>4µm C</th>
<th>4µm FA</th>
<th>30µm C</th>
<th>30µm FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{VASM}$</td>
<td>0.52</td>
<td>0.58</td>
<td>0.61</td>
<td>0.69</td>
<td>0.77</td>
<td>0.81</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.28</td>
<td>0.28</td>
<td>0.33</td>
<td>0.33</td>
<td>0.39</td>
<td>0.33</td>
</tr>
<tr>
<td>$V_{VOther}$</td>
<td>0.28</td>
<td>0.23</td>
<td>0.16</td>
<td>0.17</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>1.08</td>
<td>1.09</td>
<td>1.10</td>
<td>1.19</td>
<td>1.26</td>
<td>1.23</td>
</tr>
</tbody>
</table>

The volume fractions of smooth muscle, extracellular matrix and other elements in large transverse airways sections of 0.5µm, 4µm and 30µm thickness from a control subject and a case of fatal asthma. 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

3.3.4.2 Effect of Section Site Along a Length of Airway

The variation of $V_{VASM}$ along airway segments was <10% in all airways (Table 3.2).

3.3.4.3 Effect of Airway Size

$V_{VASM}$ tended to decrease with increased airway size and was significantly decreased in the large airways compared with small airways in the control subjects (Table 3.3).

3.4 Discussion

This study examined the sources of variability that may be encountered within and between airway segments when measuring ASM parameters. To understand the mechanisms of remodelling of the ASM layer in airway disease, 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 volume density of ASM cells. We found that estimated area of ASM was increased with section thickness, most notably in 30μm, and varied by as much as 30% along large airway segments. The volume density of ASM cells was best estimated using 40 random high-power fields and varied little around airway segments, and by <22% between airways in the same subject. We also found that volume fractions of tissue components were optimally estimated on 0.5μm 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 (James and Carroll 2000) and it varies in thickness along large airways (Heard and Hossain 1973; Hossain 1973) as we found in the present study. The variation of Nv 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_{ASM} (5 - 9%) and V_{ECM} (12 - 29%) observed along airways are similar to estimates of airway dimensions (1-7%) and inflammatory cell counts (11 - 34%) reported previously (Carroll, Lehmann et al. 1996). Using standard formulae such as that of Dobson (Dobson 1984) for normally distributed continuous data, we can estimate the number of subjects required to detect differences between subject groups, based on the variability of these measures that I have observed.

The CV of the A_{ASM} layer measured on 30μm ranged from 9% to 30% along segments of large airways and was independent of the absolute value. The variation in A_{ASM} between 30μm sections within the same airway segment observed in the present study was similar to those observed in 4μm sections by Heard and Hossain (Heard and Hossain 1973; Woodruff, Dolganov et al. 2004). In the present study, variation of the measured A_{ASM} due to different section thickness was also observed. A_{ASM} increased more in thicker sections from fatal asthmatics (28.8% increase) compared with thicker sections from control subjects (12.1% increase) (Figure 3.1). This results from the increased variation in the absolute thickness of the ASM layer (rather than that relative to the mean thickness, the coefficient of variation as in Table 3.2) in cases of asthma compared with non-asthmatic subjects. The increased estimated thickness observed with
increased section thickness is due to increasing overlap with regions of increased thickness. Therefore both the site along an airway segment and the section thickness will contribute to variation in estimated $A_{ASM}$ obtained from a single airway section. We suggest that the use of 4$\mu$m or 30$\mu$m sections will reduce variation due to the differences observed in $A_{ASM}$ over very short lengths of airway within subjects, although these thicknesses may lead to a very small overestimation of average $A_{ASM}$ compared with 0.5$\mu$m sections. This bias is very small relative to the differences observed between control subjects and asthma cases.

With increased section thickness, the tissue elements within the ASM layer overlap and, in the case of the ill-defined ECM and spaces due to artefact or oedema, cannot easily be distinguished. In very thin sections, overlap of tissues is minimised 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 $V_{VASM}$ we observed in relation to section thickness would result in over-estimation of volume fractions by approximately 25% and 20% in 30$\mu$m and 4$\mu$m sections respectively. As the tissue thickness increased, the separately estimated fractions of 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 $N_V$ in that tissue (Section 2.8). In previous studies (Heard and Hossain 1973; Ebina, Yaegashi et al. 1990; Woodruff, Dolganov et al. 2004), the volume portion of $N_V$ has included ASM, ECM and other elements. This may have resulted in over-estimation of ASM cell size. The study of Ebina (Ebina, Yaegashi et al. 1990) 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$\mu$m$^3$ in control cases. This value is considerably higher than that observed in this study (2,757$\mu$m$^3$ in control cases) where ECM and other components have been excluded from volume of the ASM layer used to estimate mean ASM cell volume. ASM cell volume, estimated from biopsy samples of proximal airways (Woodruff, Dolganov et al. 2004; Regamey, Ochs et al. 2008) and not correcting for ECM, is lower than those estimated from post-
mortem tissue sections, ranging from approximately 2,000\(\mu\text{m}^3\) to 2,700\(\mu\text{m}^3\). It is possible that biopsies yield lower values for ASM cell volume due to tissue preparation artefacts or due to differences between live and post-mortem specimens. Systematic differences in the components of the ASM layer between disease cases and controls could also result in an overestimation of the degree of hypertrophy in disease.

Variability of airway components between airways of different size is important since examination of airway pathology in most patients is necessarily confined to biopsy of the large (central) airways. We found that \(V_{\text{VASM}}\) 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). 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 remodelling changes in the ASM layer of small airways based on findings from biopsies of the large airways should be undertaken with caution.

The fixation in formalin and embedding in paraffin used in the present study is excellent for preservation of structure, however it results in unpredictable tissue shrinkage (Lum and Mitzner 1985). While 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. Shrinkage does increases the potential for variation (more noise) or bias (less accuracy) when comparing tissues from different lungs. Another technical consideration 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\(\mu\text{m}\)) airway sections. The volume fractions of each ECM component would then need to be totalled and compared with an airway section stained with the Masson’s Trichrome technique.
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 $V_{VASM}$. ASM parameters should be estimated on both large and small airways in order to account for the differences between airway sizes. The findings from these experiments have formed the basis for the methods used in subsequent chapters. That is, that 40 or more high-power fields were used to estimate ASM cell numerical density, volume fractions were estimated using 0.5µm sections and the area of the ASM layer was estimated using 4µm or 30µm thick sections. Implementing these strategies should result in accurate estimations of ASM cell parameters when assessing the effects of diseases such as asthma and COPD. In addition, knowledge of sources of variation can help estimate the number of samples required to ensure adequate power in studies comparing parameters of the layer of ASM between individuals.
4.1 Introduction

Asthma is characterised by airway hyper-responsiveness, excessive inflammation and airway remodelling, with an increase in the thickness of the ASM layer a major component of this remodelling (James 1997). Mathematical models suggest that ASM thickening may be the primary contributor to airway hyper-responsiveness (Wiggs, Bosken et al. 1992; Lambert, Wiggs et al. 1993; Oliver, Fabry et al. 2007) although the main mechanism responsible for the increase in the ASM layer thickness is unclear. Increased thickness of the ASM layer in asthma may result from hyperplasia or hypertrophy of muscle cells, increased ECM or a combination of both. Although numerous studies have been undertaken to determine whether the increased thickness of the ASM layer in asthma is due to hypertrophy (Benayoun, Druiilhe et al. 2003), hyperplasia (Heard and Hossain 1973; Woodruff, Dolganov et al. 2004), or a combination of both (Ebina, Takahashi et al. 1993), these findings are inconsistent. Reports of hyperplasia are more frequent than those of hypertrophy however study limitations cannot be ignored. Such limitations include low subject numbers, restriction of the studies to fatal asthma or the use of biopsy samples rather than whole transverse airway segments. Most importantly, in all studies to date the proportion of ECM within ASM bundles has not been accounted for when estimating ASM cell volume. Failing to take into account the contribution of ECM to the smooth muscle layer will affect estimated cell volume and hence the determination of ASM cell hypertrophy. The aim of this study was to examine the mechanisms responsible for the increase in the area of the ASM layer in asthma (hypertrophy and/or hyperplasia) while taking into account the contribution of the ECM, and to relate findings to subject characteristics, such as age, duration and severity of asthma.

4.2 Methods

4.2.1 Subjects

Large and small airways from post-mortem lungs obtained from Perth (n = 41), Melbourne (n = 6), Sydney (n = 23), Auckland (n = 14), Calgary (n = 66) and Brazil (n = 5) were examined (Section 2.2).
4.2.2 Structural Assessment
This chapter examines ASM hypertrophy and hyperplasia in large and small airways in a large number of controls, non-fatal and fatal asthma cases. We have used thin sections to estimate the volume fractions ($V_V$) of ASM cells and ECM within the smooth muscle layer and to correct for this in the determination of ASM cell size and number. ASM cell numbers ($N_V$) were determined on 30µm sections using the optical disector approach (Section 2.7). The volume fractions of ASM cells and ECM were estimated on 0.5µm sections using the point counting technique (Section 2.6). The number of cells per mm length of airway ($N_L$) and mean ASM cell volume ($V_C$) corrected for the volume fraction of ASM ($V_{VASM}$) were estimated (Section 2.8).

4.2.3 Statistics
Airways were grouped by size for all analyses as small (Pbm <4mm), medium (Pbm 4 - 10mm) or large (Pbm >10mm). Parameters from subjects with multiple airways were assessed using subject means. The effects of centre, subject group (control, non-fatal or fatal asthma), age, sex, airway size, smoking, height, and weight were examined by multiple linear regression models (SPSS).

4.3 Results

4.3.1 Subjects
The number of subjects used per subject group (control, non-fatal asthma, fatal asthma) and subject demographics are presented in Table 4.1. Subject groups were similar, apart from weight, which was greater in the non-fatal asthma group. Severity of asthma, determined from patient symptoms, frequency and type of medication use, and prior hospitalisation, was greater in the cases of fatal asthma. Reported ages of onset and duration of asthma were similar between the fatal asthma and non-fatal asthma cases. There were no overt differences in the history of smoking between groups.
Table 4.1: Subject characteristics.

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Gender</th>
<th>Age</th>
<th>Weight</th>
<th>Height</th>
<th>Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 51)</td>
<td>M / F</td>
<td>34 / 17</td>
<td>33 ± 17</td>
<td>69 ± 17</td>
<td>172 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 / 12 / 3 / 19</td>
</tr>
<tr>
<td>Non-fatal asthma (n = 49)</td>
<td></td>
<td>27 / 22</td>
<td>29 ± 15</td>
<td>85 ± 37</td>
<td>169 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15 / 19 / 4 / 11</td>
</tr>
<tr>
<td>Fatal asthma (n = 55)</td>
<td>31 / 24</td>
<td>35 ± 18</td>
<td>71 ± 16</td>
<td>170 ± 11</td>
<td>11 / 17 / 4 / 23</td>
</tr>
</tbody>
</table>

4.3.2 Area of the ASM layer (A<sub>ASM</sub>)

A<sub>ASM</sub>, relative to airway size (A<sub>ASM</sub>/Pbm) was increased in cases of fatal (p <0.001) and non-fatal asthma (p = 0.006) compared with control subjects, and in fatal cases of asthma compared with non-fatal cases of asthma (p <0.001) (Table 4.2). These differences were also observed when examined by airway size (Figure 4.1).

A<sub>ASM</sub> was related significantly to subject group (p = 0.002), airway size (p <0.001) and center (p = 0.034) with an interaction between center and airway size (p = 0.002) when airway sizes were combined. There was no significant effect of sex or age.

Figure 4.1: Area of the ASM layer relative to the basement membrane length (A<sub>ASM</sub>/Pbm mm<sup>2</sup>) for control, non fatal and fatal asthma cases, sorted by airway size. Results displayed as mean ± 95% CI; * p <0.05 versus control subjects; # p <0.05 versus non-fatal cases of asthma.
Table 4.2: Airway smooth muscle layer parameters in small (Pbm <4mm), medium (Pbm 4 – 10mm), large (Pbm >10mm) airways and all airways combined in asthmatic and non-asthmatic subjects.

<table>
<thead>
<tr>
<th>Airway smooth muscle layer thickness, mm (A_{ASM}/Pbm)</th>
<th>Airway Size</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All airways</td>
<td>0.025 (0.022 – 0.029)</td>
<td>0.033** (0.029 – 0.037)</td>
<td>0.079*** ### (0.063 – 0.100)</td>
</tr>
<tr>
<td></td>
<td>&lt;4mm</td>
<td>0.017 (0.013 – 0.022)</td>
<td>0.022* (0.017 – 0.030)</td>
<td>0.027**</td>
</tr>
<tr>
<td></td>
<td>4-10mm</td>
<td>0.019 (0.016 – 0.022)</td>
<td>0.024* (0.020 – 0.028)</td>
<td>0.063*** ### (0.052 – 0.077)</td>
</tr>
<tr>
<td></td>
<td>&gt;10mm</td>
<td>0.043 (0.029 – 0.062)</td>
<td>0.061** (0.042 – 0.089)</td>
<td>0.089*** ## (0.064 – 0.123)</td>
</tr>
</tbody>
</table>

Mean and 95% confidence intervals adjusted for age, sex, center and airway size (Pbm). * = p <0.05 vs. control; ** = p <0.01 vs. control; *** = p <0.001 vs. control; # = p <0.05 vs. non-fatal asthma; ## = p <0.01 vs. non-fatal asthma; ### = p <0.001 vs. non-fatal asthma.

4.3.3 Volume Fraction of ASM and ECM

Compared with control subjects, V_{VASM} was increased in cases of fatal asthma (p = 0.008), but not in cases of non-fatal asthma (p = 0.492) (Figure 4.2, Table 4.3). Compared with cases of non-fatal asthma, V_{VASM} was increased in cases of fatal asthma (p = 0.034) and this effect was confined to the large airways (p = 0.018). There were significant effects on V_{VASM} for subject group (p = 0.023), but not for sex, center, age or airway size, when airway sizes were combined.

For V_{VECM}, when airway sizes were combined, there was a significant effect for subject group (p = 0.025) but not for sex, center, age or airway size. There were no significant differences between individual subject groups (Table 4.3). V_{VECM} was decreased in the large airways of the fatal asthma subjects compared with non-fatal asthma and controls (Figure 4.3). There were no significant effects on other components (V_{Vother}) within the layer of ASM for all airways combined (Table 4.3). V_{Vother} was significantly greater in the medium sized airways in the non-fatal and fatal cases of asthma compared with controls.
Table 4.3: Volume fractions of ASM, ECM and other within the ASM layer in small (Pbm <4mm), medium (Pbm 4 – 10mm), large (Pbm >10mm) airways and all airways combined in asthmatic and non-asthmatic subjects.

<table>
<thead>
<tr>
<th>Airway Size</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>All airways</td>
<td>0.64 (0.60 – 0.68)</td>
<td>0.65 (0.61 – 0.69)</td>
<td>0.67** #</td>
</tr>
<tr>
<td>&lt;4mm</td>
<td>0.69 (0.66 – 0.72)</td>
<td>0.69 (0.65 – 0.72)</td>
<td>0.69</td>
</tr>
<tr>
<td>4 – 10mm</td>
<td>0.67 (0.64 – 0.70)</td>
<td>0.65 (0.62 – 0.68)</td>
<td>0.66</td>
</tr>
<tr>
<td>&gt;10mm</td>
<td>0.61 (0.57 – 0.65)</td>
<td>0.63 (0.59 – 0.66)</td>
<td>0.65*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Airway Size</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>All airways</td>
<td>0.19 (0.18 – 0.20)</td>
<td>0.18 (0.17 – 0.19)</td>
<td>0.19</td>
</tr>
<tr>
<td>&lt;4mm</td>
<td>0.20 (0.18 – 0.23)</td>
<td>0.20 (0.17 – 0.23)</td>
<td>0.19</td>
</tr>
<tr>
<td>4 – 10mm</td>
<td>0.17 (0.16 – 0.18)</td>
<td>0.17 (0.15 – 0.18)</td>
<td>0.16</td>
</tr>
<tr>
<td>&gt;10mm</td>
<td>0.19 (0.17 – 0.22)</td>
<td>0.19 (0.16 – 0.21)</td>
<td>0.16* ##</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Airway Size</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>All airways</td>
<td>0.15 (0.12 – 0.17)</td>
<td>0.15 (0.12 – 0.17)</td>
<td>0.15</td>
</tr>
<tr>
<td>&lt;4mm</td>
<td>0.12 (0.05 – 0.20)</td>
<td>0.13 (0.05 – 0.20)</td>
<td>0.14</td>
</tr>
<tr>
<td>4 – 10mm</td>
<td>0.14 (0.12 – 0.16)</td>
<td>0.17* (0.15 – 0.18)</td>
<td>0.18**</td>
</tr>
<tr>
<td>&gt;10mm</td>
<td>0.20 (0.17 – 0.23)</td>
<td>0.19 (0.16 – 0.22)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Mean and 95% confidence intervals adjusted for age, sex, center and airway size (Pbm).

* = p <0.05 vs. control; ** = p <0.01 vs. control; *** = p <0.001 vs. control; # = p <0.05 vs. non-fatal asthma; ## = p <0.01 vs. non-fatal asthma; ### = p <0.001 vs. non-fatal asthma.
**4.3.4 Numerical Density \( (N_V) \) and Mean Volume of ASM Cells \( (V_C) \)**

Compared with control subjects, \( N_V \) was decreased in cases of fatal asthma \( (p < 0.001) \) and in cases of non-fatal asthma \( (p = 0.002) \) (Figure 4.4, Table 4.4). When airway sizes were combined, there were significant effects on \( N_V \) for subject group \( (p = 0.003) \) and center \( (p = 0.031) \), but not airway size, sex or age.

\( V_C \) was increased in males compared with females \( (p = 0.008) \). Compared with control subjects, \( V_C \) was increased in cases of fatal asthma \( (p = 0.005) \) and non-fatal asthma (p
which were similar to each other (Figure 4.5, Table 4.4). The increase in $V_C$ was in the large airways in both fatal asthma ($p < 0.001$) and non-fatal asthma ($p = 0.015$). There were significant effects on $V_C$ for subject group ($p=0.003$), sex ($p = 0.025$) and center ($p < 0.001$), but not for airway size or age when airway sizes were combined.

Table 4.4: Numerical density and volume of ASM cells in small (Pbm <4mm), medium (Pbm 4 – 10mm), large (Pbm >10mm) airways and all airways combined in asthmatic and non-asthmatic subjects.

<table>
<thead>
<tr>
<th>Airway Size</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>All airways</td>
<td>2.738 (2.550 – 2.966)</td>
<td>2.333** (2.168 – 2.413)</td>
<td>2.221*** (2.042 – 2.413)</td>
</tr>
<tr>
<td>&lt;4mm</td>
<td>3.038 (2.547 – 3.626)</td>
<td>2.697 (2.255 – 3.229)</td>
<td>2.583 (2.277 – 2.930)</td>
</tr>
<tr>
<td>4 - 10mm</td>
<td>2.878 (2.568 – 3.193)</td>
<td>2.452* (2.210 – 2.724)</td>
<td>2.138*** (1.897 – 2.413)</td>
</tr>
<tr>
<td>&gt;10mm</td>
<td>2.380 (2.206 – 2.583)</td>
<td>2.028* (1.870 – 2.192)</td>
<td>1.657*** ## (1.418 – 1.939)</td>
</tr>
</tbody>
</table>

| Airway smooth muscle cell volume ($V_C$), $\mu$m$^3$, x10$^3$ |
|----------------|-------------|---------------|---------------|
| All airways | 2.757 (2.550 – 2.962) | 3.118* (2.916 – 3.320) | 3.257** (2.983 – 3.532) |
| <4mm | 2.419 (1.979 – 2.859) | 2.695 (2.248 – 3.141) | 2.569 (2.183 – 2.953) |
| 4 - 10mm | 2.863 (2.416 – 3.310) | 3.104 (2.662 – 3.546) | 3.225 (2.833 – 3.616) |

Mean and 95% confidence intervals adjusted for age, sex, center and airway size (Pbm). * = $p < 0.05$ vs. control; ** = $p < 0.01$ vs. control; *** = $p < 0.001$ vs. control; # = $p < 0.05$ vs. non-fatal asthma; ## = $p < 0.01$ vs. non-fatal asthma; ### = $p < 0.001$ vs. non-fatal asthma.
Figure 4.4: Numerical density of ASM cells ($N_V$, cells/mm$^3$) for control, non fatal and fatal asthma cases, sorted by airway size. Results displayed as mean ± 95% CI; * p <0.05 versus control subjects; # p <0.05 versus non-fatal cases of asthma.

Figure 4.5: Mean volume of ASM cells ($V_C \mu m^3$) for control, non fatal and fatal asthma cases, sorted by airway size. Results displayed as mean ± 95% CI; * p <0.05 versus control subjects; # p <0.05 versus non-fatal cases of asthma.

4.3.5 Number of Cells per Unit Length of Airway

Compared with control subjects, $N_L$ was increased in cases of fatal asthma (p <0.001), but not in cases of non-fatal asthma (p = 0.086) (Figure 4.6, Table 4.5). $N_L$ was increased in cases of fatal asthma in all airway sizes. $N_L$ was also increased in cases of fatal asthma compared with cases of non-fatal asthma for airway sizes combined (p <0.001); in medium (p <0.001); and large (p = 0.003) airways. There were significant effects on $N_L$ for subject group (p = 0.025), airway size (p <0.001) and center (p =
0.013), but not for sex or age when airway sizes were combined. There was a significant interaction between center and airway size (p<0.001).

Table 4.5: Number of ASM cells per airway length in small (Pbm <4mm), medium (Pbm 4 – 10mm), large (Pbm >10mm) airways and all airways combined in asthmatic and non-asthmatic subjects.

<table>
<thead>
<tr>
<th>Airway Size</th>
<th>Control</th>
<th>Non-fatal asthma</th>
<th>Fatal asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N₀L/mm) x10⁵</td>
<td>(N₀L/mm) x10⁵</td>
<td>(N₀L/mm) x10⁵</td>
</tr>
<tr>
<td>All airways</td>
<td>0.611 (0.523–0.712)</td>
<td>0.731 (0.629–0.845)</td>
<td>1.670*** ###</td>
</tr>
<tr>
<td>&lt;4mm</td>
<td>0.134 (0.099–0.181)</td>
<td>0.165 (0.122–0.224)</td>
<td>0.189*</td>
</tr>
<tr>
<td>4–10mm</td>
<td>0.339 (0.287–0.398)</td>
<td>0.374 (0.321–0.439)</td>
<td>0.846*** ###</td>
</tr>
<tr>
<td>&gt;10mm</td>
<td>1.297 (0.899–1.872)</td>
<td>1.539 (1.060–2.235)</td>
<td>2.293*** ##</td>
</tr>
</tbody>
</table>

Mean and 95% confidence intervals adjusted for age, sex, center and airway size (Pbm). * = p <0.05 vs. control; ** = p <0.01 vs. control; *** = p <0.001 vs. control; # = p <0.05 vs. non-fatal asthma; ### = p <0.001 vs. non-fatal asthma.

Figure 4.6: Number of ASM cells per length of airway (N₀L/mm) for control, non-fatal and fatal asthma cases, sorted by airway size. Results displayed as mean ± 95% CI; * p <0.05 versus control subjects; # p <0.05 versus non-fatal cases of asthma.
4.3.6 Effects of Age and Duration of Asthma

When both fatal asthma and non-fatal asthma were combined there were no significant effects of age on ASM cell number or volume and only a small but significant effect on V\textsubscript{Vother} (p = 0.039). The duration of asthma had a small positive effect, independent of age, on the A\textsubscript{ASM}/Pbm (p = 0.012) and N\textsubscript{L} (p = 0.042, Figure 4.7) in large airways. Within asthma groups, there was a significant effect of age on A\textsubscript{ASM}/Pbm in large airways of cases of fatal asthma (p = 0.05) and of duration of asthma on A\textsubscript{ASM}/Pbm in the large airways of cases of non-fatal asthma (p = 0.018) but no effects of age or duration of asthma on ASM cell number (Figure 4.7) or size (Figure 4.8) or on tissue fractions within the ASM layer.

![Figure 4.7: The number of ASM cells per unit length of airway (N\textsubscript{L}) in relation to the duration of asthma, in cases of non-fatal asthma (--- r = 0.082, p = 0.656) and fatal asthma (— r = 0.034, p = 0.862).](image-url)
Figure 4.8: The average ASM cell volume ($V_C$) in relation to the duration of asthma, in cases of non-fatal asthma (--- $r = 0.089$, $p = 0.626$) and fatal asthma (—— $r = 0.195$, $p = 0.321$).

**4.3.7 Relation to Subject Characteristics**

History of smoking cigarettes, height and body weight did not have independent effects on any ASM parameters. There was insufficient numbers for meaningful analysis of the effects of various medications. We did observe that the cases from Sao Paulo had very low corticosteroid use (both inhaled and oral) although the trends in these cases were not systematically different from any other centre.

**4.4 Discussion**

The study shows that when adjusted for study center, age, sex, airway size and the contribution of ECM, the increased thickness of the layer of ASM in asthma is due to hyperplasia of ASM cells in the large, medium and small airways in fatal cases of asthma, and to hypertrophy of ASM cells in the large airways in cases of both fatal and non-fatal asthma. Significant hyperplasia was not observed in cases of non-fatal asthma.

In general, there were no significant changes in the volume fraction of ECM or muscle in the ASM layer between subject groups suggesting that there was a concurrent increase in the amount of tissue matrix along with the increase in ASM cell size and
number. While multiple regression analysis suggested an effect of subject group on volume fractions, this is likely dominated by changes in the large airways of fatal cases of asthma where the fraction of smooth muscle was increased and the fraction of ECM was decreased. The significance achieved in the large airways of fatal asthma is consistent with the trend towards an increase in the volume fraction of ASM observed in the previous chapter, which was likely not significant due to the small sample size. These findings suggest that asthma (mild, moderate or severe) is characterised by ASM cell hypertrophy in the large airways and that fatal asthma (predominantly severe) is characterised by hyperplasia of ASM cells in both the large and small airways. Since clinical data were not available for all subjects and clinical severity was strongly associated with asthma group, we could not detect an independent effect of clinical severity. Both hypertrophy and hyperplasia of ASM were accompanied by an increase in ECM such that the volume fractions of muscle and matrix were generally similar in asthmatic and non-asthmatic airways.

There were no significant effects of age on ASM cell number or size, relative to the effect of asthma group, although an increased duration of asthma was associated with a small increase in ASM cell number in cases of asthma. This suggests that the increase in ASM cell number or size is predominantly determined early in life or early in the course of asthma. Moreover, it seems likely that ASM cell number may contribute to the clinical severity of asthma. Events subsequent to the onset of asthma may result in a small increase in the number of ASM cells, but have little effect on the size of ASM cells. Previous longitudinal studies of asthma (relative to control subjects) show little change in lung function (Phelan, Robertson et al. 2002; Sears, Greene et al. 2003; James, Palmer et al. 2005) or asthma severity (Phelan, Robertson et al. 2002) over time, with structural changes, especially area of ASM shown to be related to airflow obstruction in children (Tillie-Leblond, de Blic et al. 2008). These observations, in addition to our own, suggest that the structural changes in the asthmatic airway occur early in the natural history of asthma, principally in childhood (Pohunek, Warner et al. 2005; Saglani, Payne et al. 2007).

A number of previous studies have examined ASM hyperplasia and hypertrophy in asthma (Heard and Hossain 1973; Ebina, Takahashi et al. 1993; Woodruff, Dolganov et al. 2004; Regamey, Ochs et al. 2008). The current study shows that hyperplasia is
present in cases of clinically severe, fatal asthma. This agrees with the observations of Heard and Hossain (Heard and Hossain 1973) and Ebina et al. (Ebina, Takahashi et al. 1993) who compared cases of fatal asthma with non-asthmatic individuals. Although there was a trend in the large airways (p = 0.086), we did not find a statistically significant increase in the number of ASM cells per length of airway in cases of non-fatal asthma. This agrees with the findings of Regamey et al. (Regamey, Ochs et al. 2008) who found hyperplasia in cases of moderate-severe asthma, similar to the clinical severity of asthma in our cases of fatal asthma. Our results contrast with the study of Woodruff et al. (Woodruff, Dolganov et al. 2004) who reported hyperplasia in cases of mild-moderate asthma. In that study, biopsies of central airways were examined whereas in the present study, the large airways included all cartilaginous airways, many of which would not normally be accessible to biopsy by bronchoscope. In addition, our cases of non-fatal asthma were from the general community, unlike the clinic patients in other studies (Woodruff, Dolganov et al. 2004; Regamey, Ochs et al. 2008) and therefore likely to be less severe. Taken together, the results of all studies suggest a relation between the number of ASM cells in the central airways and the clinical severity of asthma.

We found that hypertrophy was present in the large airways of cases of both fatal and non-fatal asthma. Previous studies have observed hypertrophy in post-mortem studies of whole airway sections from fatal cases of asthma, (Ebina, Takahashi et al. 1993) but not in biopsies from less severe cases of asthma (Woodruff, Dolganov et al. 2004; Regamey, Ochs et al. 2008). In biopsy studies, only a small sample of tissue is obtained for examination. Estimation of the mean ASM cell volume is based on the density of ASM cells per volume of tissue. Due to the small volume of tissue obtained and the variability of tissue fractions between biopsies, it is possible that the size of bronchoscopic biopsies is inadequate to detect ASM hypertrophy in these cases.

The fraction of the ASM layer that was occupied by ECM in the present study was similar in the asthma cases and control subjects (despite the increase in cell number and/or size), except in the large airways in cases of fatal asthma where it was decreased and the volume fraction of ASM was increased. That is, the increased absolute volume of muscle within the ASM layer was matched by an increase in the absolute volume of ECM, albeit to a lesser extent in the large airways in cases of fatal asthma. The findings
in cases of fatal asthma agree with those of Bai et al. (Bai, Cooper et al. 2000) who showed that there was an increase in the area of both muscle and ECM, and an increase in the fraction of ASM in the airway wall in cases of fatal asthma compared with control non-asthmatic subjects. It has been postulated that ECM within the airway wall in asthma may act to protect the airway from excessive narrowing (McParland, Macklem et al. 2003). If the mechanical properties of the ECM and its interaction with the ASM are unchanged, then the observed reduction in the $V_{VECM}$ in the large airways of cases of fatal asthma suggests that this mechanism is unlikely.

In summary, we have observed hypertrophy in the non-fatal (predominantly moderate severity) and fatal cases of asthma and hyperplasia in the fatal cases (predominantly severe) both of which should increase airway narrowing capacity. Both moderate and severe cases of asthma are characterised by excessive airway narrowing. Increased total contractile proteins, either by hypertrophy or by hyperplasia results in more force development and excessive airway narrowing, as suggested by Oliver et al. (Oliver, Fabry et al. 2007). The functional effects of ASM hypertrophy and hyperplasia in asthma are currently open to speculation. Determining the functional implications of a thickened ASM layer in airways from asthmatic individuals forms the basis of experiments presented in the following chapter.
CHAPTER 5: Airway Narrowing and Bronchodilation to Deep Inspiration in Bronchial Segments from Asthmatic Subjects

5.1 Introduction

The primary functional abnormality observed in asthma is airway hyperresponsiveness, particularly excessive airway narrowing, to bronchoconstricting stimuli. However our understanding of the mechanism(s) producing this excessive airway narrowing remains unclear. Another functional characteristic of subjects with asthma is the failure of a DI to induce bronchodilation, a process which normally occurs in non-asthmatic subjects (Nadel and Tierney 1961; Brusasco, Crimi et al. 1999; Crimi, Pellegrino et al. 2002; Salome, Thorpe et al. 2003). The loss of the beneficial bronchodilatory response to DI may contribute to airway pathophysiology in asthma (Skloot, Permutt et al. 1995; Skloot and Togias 2003) and has even been suggested as the main cause of excessive airway narrowing (Fish, Ankin et al. 1981). The interrelationship between the failure of the DI response and airway hyperresponsiveness in asthma remain unclear.

Airway remodelling, particularly the increased mass of ASM, is also likely to play an important role in the pathogenesis of airway hyperresponsiveness in subjects with asthma. In Chapter 4 it was shown that the increased thickness of ASM layer in asthma results from both hypertrophy and hyperplasia of ASM cells, as well as an increase in the ECM. Mathematical models have shown a relationship between ASM mass and maximal airway narrowing, however this is based on the assumption that maximal force production by the remodelled ASM is also increased (Affonce and Lutchen 2006; Lambert, Wiggs et al. 1993). General remodelling of other wall compartments may also contribute to increased airway narrowing (Lambert, Wiggs et al. 1993) but this may be offset by alterations in airway wall stiffness, increasing the load on the ASM (McParland, Macklem et al. 2003). Whether structural or mechanical abnormalities of the airway wall contribute to the reduced bronchodilatory response to DI in asthma is not clear.

The main aim of this chapter was to determine whether structural remodelling of the airways in asthma, particularly thickening of the ASM layer, increases the narrowing capacity of the airway and contributes to exaggerated airway narrowing. A secondary
aim was to determine whether the reduced bronchodilatory response to normal breathing movements is related to a reduced response of the airway wall to mechanical stress/strain and how this is impacted by remodelling. It was hypothesised that airways from subjects with a history of asthma would narrow more than those without asthma and that this would be related to an increase in ASM mass.

5.2 Methods

5.2.1 Subjects
Subjects were recruited from Sir Charles Gairdner Hospital and the Mount Hospital several days prior to surgery for the removal of pulmonary neoplasms. Non-asthmatic (control) and asthmatic subjects were recruited for this study. Subjects were defined as having a history of asthma by a positive response to the question “Has your doctor ever told you that you have asthma?”. Standard lung function, a respiratory questionnaire and allergy tests to common aero-allergens were performed on all patients (Section 2.3).

5.2.2 Experimental Protocol
Isolated bronchial segments were collected post-operatively and mounted in a custom-made organ bath system for measurement of airway mechanics in vitro. Intraluminal pressure was controlled and adjusted to simulate the normal loads present in the airway wall including those from the dynamic breathing environment. (Section 2.9 and 2.10)

Cumulative dose-response curves to acetylcholine (ACh) (3x10^{-6} M to 3x10^{-3} M) were performed under static conditions with transmural pressure held fixed at 5cmH_{2}O, and dynamic conditions simulating tidal oscillations with intermittent DI (the order of protocols was randomised). Airway narrowing (Δ volume, %) dose-response curves to ACh was measured during simulated tidal and DI oscillations. DI was administered once airway narrowing to the prior dose had stabilised. The dynamic compliance of the airways was measured from volume and pressure cycles during tidal oscillation and normalised to the absolute airway volume measured at the end of the experiment. Bronchodilatory response to theophylline was also assessed. The response to DI under comparable levels of baseline airway narrowing was also examined in a subset of airways. Airways were narrowed to varying doses of carbachol to match the level of airway narrowing of the control and asthmatic airways prior to the application of DI.
5.2.3 Structural Assessment

To assess airway structure, consecutive transverse airway sections were cut at 0.5μm, 4μm and 30μm. The volume fractions of ASM, ECM and other (V_{VASM}, V_{VECM}, V_{VOther} respectively) within the ASM layer were estimated on 0.5μm sections stained using the Masson’s Trichrome technique. The area of the ASM layer (A_{ASM}), perimeter and thickness of the basement membrane, inner wall area, outer wall area and ASM area were measured on 4μm sections stained with haematoxylin and eosin. ASM cell numerical density (N_v) and mean ASM cell volume (V_c) and the number of ASM cells per airway length (N_L) were estimated on 30μm sections (haematoxylin) using stereological techniques (Section 2.7).

5.2.4 Statistics

Maximum airway narrowing and all morphological parameters were compared between groups with and without a history of asthma using unpaired t-tests. Comparisons between bronchodilation to DI (% reversal), DI volume strains, specific compliance at baseline and the effects of tidal oscillations and DI on airway narrowing were made by two-way ANOVA and Newman-Keuls post-hoc analyses. The relationships between variables were examined with Pearson’s correlation. In vivo lung function was compared between groups using unpaired t-tests.

5.3 Results

5.3.1 Subjects

The number of subjects (control and history of asthma), demographics and lung function are presented in Tables 5.1 and 5.2. There were no differences in either pre- or post-bronchodilator % predicted FEV_{1} (p = 0.30 and 0.73) and FVC (p = 0.66 and 0.24), or FEV_{1}/FVC ratio (p = 0.16 and 0.17) between control and asthmatic subjects. One subject with asthma had a positive bronchodilator response. The mean increase in FEV_{1} after bronchodilator was 2.5 ± 2.3% in the control group and 10.6 ± 4.6% in the asthmatic group (p = 0.10). Eleven out of the thirteen subjects reported one or more respiratory symptom including breathlessness, cough, phlegm, wheeze or chest tightness. Two subjects in control group had no prior respiratory symptoms whereas as all subjects with a history of asthma experienced prior symptoms and two subjects were previously hospitalised with asthma. Of the control subjects, four were not taking any
medication, none were using inhaled respiratory medications and four were using a variety of cardiovascular, anti-hypertensive and antidepressant medications. One control subject was also on an anticholinergic medication for a bladder condition. The asthmatic subjects were using a variety of inhaled corticosteroids, long/short acting β-agonists and anticholinergic medications in addition to cardiovascular medications. Resected pulmonary neoplasms included primary carcinoids, adenocarcinomas, squamous cell carcinomas, and two secondary cancers (gastrointestinal tract and bone). In one subject, surgery was to treat bronchial atresia which was confined to a localised area within the left upper lobe. All tissues used were macroscopically normal and acquired away from the affected area of the lung.

Table 5.1: Subject characteristics

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Smoking (Y / N / Ex)</th>
<th>Pack years</th>
<th>Atopy (Y / N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 / 2</td>
<td>64 ± 15</td>
<td>169 ± 9</td>
<td>77 ± 15</td>
<td>0 / 5 / 3</td>
<td>9.5 ± 19</td>
<td>0 / 8</td>
</tr>
<tr>
<td>History of asthma</td>
<td>1 / 4</td>
<td>57 ± 21</td>
<td>165 ± 8</td>
<td>82 ± 14</td>
<td>0 / 2 / 3</td>
<td>39.2 ± 61</td>
<td>2 / 3</td>
</tr>
</tbody>
</table>

Table 5.2: Subject lung function

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>FEV$_1$ (% pred)</th>
<th>FVC (% pred)</th>
<th>FEV$_1$/FVC</th>
<th>FEV$_1$ (% pred)</th>
<th>FVC (% pred)</th>
<th>FEV$_1$/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83.7 ± 19.0</td>
<td>82.9 ± 17.3</td>
<td>0.75 ± 0.07</td>
<td>85.6 ± 19.5</td>
<td>82.6 ± 18.9</td>
<td>0.78 ± 0.08</td>
</tr>
<tr>
<td>History of asthma</td>
<td>74.1 ± 10.0</td>
<td>88.4 ± 14.7</td>
<td>0.66 ± 0.14</td>
<td>81.9 ± 16.8</td>
<td>94.2 ± 10.1</td>
<td>0.68 ± 0.15</td>
</tr>
</tbody>
</table>

5.3.2 Airway Structure

There was no difference in the length (p = 0.97), volume (p = 0.79) or size (Pbm, p = 0.64) of airway segments between the control and asthmatic groups. Pbm was 11.5 ± 1.2mm (6.6 – 16.7) in airways from control subjects and 10.6 ± 1.7mm (4.8 – 14.2) in airways from asthmatic subjects. The thickness of the basement membrane was 4.3 ± 0.2µm and 6.4 ± 1.7µm in the control and asthmatic groups respectively (p = 0.28).
Area of mucous glands \( (p = 0.54) \) and area of cartilage \( (p = 0.49) \) was similar between the subject groups.

5.3.3 Airway Wall Morphometry

The \( A_{ASM} \) was increased in airways from subjects with a history of asthma (Figure 5.1). There were no significant differences in inner \( (p = 0.19) \), outer \( (p = 0.90) \) or total wall areas \( (p = 0.80) \) between groups. \( A_{ASM} \) within the airway wall was increased in subjects with a history of asthma compared with control subjects (Figure 5.2, \( p = 0.02 \)).

Figure 5.1: Histological images from a control (left) and an asthmatic subject (right). The area of ASM is indicated by the arrows. The basement membrane perimeter in the non-asthm

Figure 5.2: The area of the ASM layer per length of basement membrane \( (A_{ASM}/P_{bm}) \) in airways from eight control subjects and five subjects with a history of asthma. \( *p = 0.014 \) compared with control group.
5.3.4 Volume Fractions of ASM and ECM

There were no significant differences in the V_{VASM}, V_{VECM}, or V_{VOther} in the ASM layer of airways between control and asthmatic subjects (Table 5.3).

5.3.5 Numerical Density (N_V), Mean Volume (V_C) and Number (N_L) of ASM Cells

There were no significant differences in N_L, N_V or V_C between subjects groups (Table 5.3).

Table 5.3: Structural measurements and levels of significance for asthma and control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>History of Asthma</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{VASM}</td>
<td>0.67 ± 0.02</td>
<td>0.69 ± 0.02</td>
<td>0.44</td>
</tr>
<tr>
<td>V_{VECM}</td>
<td>0.13 ± 0.02</td>
<td>0.09 ± 0.03</td>
<td>0.36</td>
</tr>
<tr>
<td>V_{VOther}</td>
<td>0.21 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>0.67</td>
</tr>
<tr>
<td>N_L (cells/mm) x 10^4</td>
<td>8.5 ± 1.7</td>
<td>11.9 ± 3.4</td>
<td>0.35</td>
</tr>
<tr>
<td>N_V (cells/mm^3) x 10^4</td>
<td>24.5 ± 2.3</td>
<td>25.3 ± 2.8</td>
<td>0.81</td>
</tr>
<tr>
<td>V_C (µm^3) x 10^3</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Abbreviations: V_{VASM}, volume fractions of ASM; V_{VECM}, volume fraction of ECM; V_{VOther}, volume fraction of all other components; N_V, numerical density; V_C, mean volume and N_L, number of ASM cells within the ASM layer.

5.3.6 In Vitro Resting Tone

Bronchodilator response to theophylline was used as an indication of intrinsic (resting) ASM tone and was present in six out of eight airways from the control group (11.3 ± 4.0% increase in volume) and three out of five airways in the asthmatic group (7.4 ± 3.5% increase in volume). There was no statistical difference in the level of intrinsic ASM tone between subject groups (p = 0.51).

5.3.7 Airway Narrowing under Static Conditions

Dose-response curves under static or dynamic conditions (during simulated tidal and DI oscillations) were performed in random order. There was no order bias in either group...
and the level of airway narrowing measured during the first dose-response curve did not differ from the second (p = 0.51).

Comparisons between airway narrowing were initially made under static conditions in the absence of simulated breathing movements. Measurements made in this manner are in the absence of applied cyclical ASM stresses and strains and are therefore not influenced by mechanisms related to ASM dynamics. Under static conditions airway narrowing to ACh was increased in bronchi from asthmatic patients (n = 5) (Figure 5.3). Maximal airway narrowing was 29.1 ± 3.6% volume in the control group (n = 8) and 51 ± 4.5% volume in the asthmatic group (p = 0.003) (Figure 5.4). Maximal airway narrowing was positively correlated with area of the ASM layer (Figure 5.5). Maximal airway narrowing was not correlated with Pbm (p = 0.67) or the areas of the inner (p = 0.16), outer (p = 0.85) and total wall (p = 0.96), cartilage (p = 0.65) or mucous glands (p = 0.98). There was a trend towards increased maximal narrowing with basement membrane thickness but this did not reach statistical significance (p=0.084). Compared with changes in maximal airway narrowing in asthma, there was no difference in airway sensitivity between groups. Airway sensitivity to ACh was 3.6 ± 0.2 and 4.1 ± 0.2 in control and history of asthma groups respectively (p = 0.14).

Figure 5.3: Airway narrowing (Δ volume, %) dose-response curves to ACh in airway segments from control and asthmatic subjects. ACh dose was administered in a cumulative fashion to the serosal surface and airway narrowing was measured under static conditions (5cmH₂O).
Figure 5.4: Maximal airway narrowing ($E_{\text{max}}$) in the control and asthmatic groups. **$p = 0.003$. 

Figure 5.5: Maximal airway narrowing ($\Delta$ volume %) in control and asthmatic airways. Airway narrowing was positively correlated with $A_{\text{ASM}}$ ($\sqrt{A_{\text{ASM}}/P_{\text{bm}}}$).

5.3.8 Airway Narrowing during Tidal and DI Oscillations

Figure 5.3 show airway narrowing measured during tidal oscillations with intermittent DI for both controls (Figure 5.7A) and subjects with a history of asthma (Figure 5.7B). Pre-DI indicates the level of airway narrowing prior to the induction of the DI. This provides some indication of the independent effects of tidal oscillations. The level of airway narrowing immediately after and one minute after the DI are indicated by DI-0
and DI-1 respectively. Airway narrowing under static conditions is also plotted for comparison. In the non-asthmatic group maximal airway narrowing prior to the induction of the DI was 24.1 ± 4.6% volume, less than that observed under static conditions (29.1 ± 3.6% volume, p = 0.0002). DI produced immediate bronchodilation and persisted for at least one minute, with maximal airway narrowing falling to 11.4 ± 3.7% volume (p = 0.0001). Maximal airway narrowing one minute after DI was 19.4 ± 4.2% volume, and was less than that observed prior to the administration of the DI (p = 0.001). In the asthmatic group, maximal airway narrowing prior to DI was 45.8 ± 7.2 % volume compared with 51.0 ± 4.5% volume under static conditions, although this was not statistically different (p = 0.35). Similarly, DI produced immediate bronchodilation in the asthmatic group, reducing airway narrowing to 32.7 ± 10.1% volume (p = 0.001). Bronchodilation was no longer evident one minute after (p = 0.21) and airway narrowing had recovered to 42.2 ± 7.6%.

Figure 5.7: The level of airway narrowing in non-asthmatic (A) and asthmatic subjects (B) immediately following (DI-0) and one minute after a DI (DI-1). Airway narrowing under static conditions and pre-DI are shown for comparisons.

Bronchodilation to DI was expressed as % reversal which indicates the amount that airway narrowing was reversed by the DI (Figure 5.8). This analysis was only performed at the three highest ACh doses since lower doses did not always produce airway narrowing. Bronchodilation to DI tended be greater in the control group (n = 8) compared with the asthmatic group (n = 5) but this was not statistically significant (p = 0.12). Bronchodilation to DI was reduced with a greater ACh dose (Figure 5.9, p = 0.006). Bronchodilation to DI was inversely correlated with airway narrowing at 3x10^{-3}.
M ACh (p = 0.02, r = -0.64: Figure 5.4B), 10^{-3} M (p = 0.03, r = -0.60) but not at 10^{-4} M ACh (p = 0.10, r = -0.47). √A_{ASM} was inversely correlated with bronchodilation to DI at 10^{-4} M (p = 0.02, r = -0.62) but not at the two highest doses (p = 0.09, p = 0.01).

Figure 5.8: Bronchodilation (% recovery) to DI in airways from control and asthmatic subjects.

Figure 5.9: Bronchodilation (% recovery) to DI in airways from control and asthmatic subjects. Bronchodilation was related to the level of airway narrowing (Δ volume, %). Data points refer to bronchodilation and airway narrowing at the highest dose of ACh (i.e. 3x10^{-3} M).
A subset of airways (n=4 per group) were examined to compare the response to DI under comparable levels of baseline airway narrowing. Airways were narrowed to varying doses of carbachol to match the level of airway narrowing of the control and history of asthma airways prior to the application of DI. Airway narrowing in the controls was 25.8 ± 8.9% volume and 29.1 ± 5.8% volume in the history of asthma group (p = 0.77). In the control group, the mode carbachol dose was 3x10^{-6}M with a highest dose of 10^{-5}M, compared with a mode dose of 10^{-6}M with a highest dose of 3x10^{-6}M in the asthmatic group. Figure 5.10A shows the relationship between bronchodilation (% reversal) and three different sized DIs (15, 30 and 60cmH_{2}O) in a subset of airways from control (n = 4) and asthmatic subjects (n = 4). As expected, the bronchodilatory response and the volume strain increased with the amplitude of DI (p = 0.0001) but there was no difference in the amount of bronchodilation produced between the control (p = 0.76 and history of asthmatic groups (p = 0.98). The volume strains during DI, that is the expansion of the airway during the DI, were also comparable between groups (Figure 5.10B).

Figure 5.10: Relationship between the amplitude of DI (peak pressure, cmH_{2}O) and bronchodilation after DI (% reversal, A), and volume strain (Δ volume %, B) during DI.

5.3.9 Compliance, Collapsibility and Volume Strains

The dynamic compliance of the airways was measured from volume and pressure cycles during tidal oscillation and normalised to the absolute airway volume measured at the end of the experiment. The baseline compliance (prior to ACh) of airways was 0.019 ± 0.004 cmH_{2}O^{-1} in the control group and 0.028 ± 0.009 cmH_{2}O^{-1} in the history of asthma group which were not statistically different (p = 0.37). ACh produced a reduction in
compliance in both groups (p = 0.0002) which often reached maximum stiffening at least one dose earlier than that required for maximal airway narrowing. No difference in airway compliance was observed under conditions of maximal stiffening between the control (0.015 ± 0.004cmH$_2$O$^{-1}$) and asthmatic groups (0.020 ± 0.008cmH$_2$O$^{-1}$). The pressure required to collapse the airway was -18.1 ± 8.4cmH$_2$O in the control group and -9.0 ± 3.8cmH$_2$O in the asthmatic group, although these were not statistically different (p = 0.44).

The compliance (baseline or after contraction with ACh) and collapsibility of airways were unrelated to any morphological parameter except $N_L$ which was positively correlated with both baseline (p = 0.02, r = 0.63) and contracted (p = 0.01, r = 0.67) compliance. This relationship was dominated by a single highly compliant airway from the history of asthma group and significance was lost once the outlier was removed (p = 0.62 and p = 0.34). Compliance prior to administration of DI did not correlate with % reversal (i.e. bronchodilation) at any of the three highest ACh doses. Maximal airway narrowing (static) was not related to baseline (p = 0.087) or contracted compliance (p = 0.079), or pressure to collapse (p = 0.13). These results were not altered when the outlier was removed.

At baseline (prior to ACh) volume strain was 9.1 ± 1.6% during tidal oscillations and 37.9 ± 7.6% during DI in the control group, and 9.7 ± 1.2% during tidal oscillations and 45.2 ± 6.4% in the history of asthma group. For these calculations the highly compliant airway in the asthmatic group was excluded since it was outside three times the standard deviation of the mean. There was no statistical difference in the volume strains between control and asthmatic groups.

5.4 Discussion

This study found that the maximal narrowing response was correlated with the area of the ASM layer and that airways from subjects with a prior history of asthma have an increased capacity to narrow. Taken together these findings suggest that airway hyperresponsiveness present in subjects with asthma partly involves an abnormality specific to the airway wall and that the amount of ASM impacts the presence or absence of airway hyperresponsiveness. The study also showed that at the isolated airway level,
the capacity for normal breathing movements to dilate the airway was not altered in subjects with a prior history of asthma. This suggests that impairment in the response of the airway wall to cyclical mechanical stresses and strain may not explain airway hyperresponsiveness.

The major restriction to studies examining the behaviour of human airways in vitro is access to suitable tissue. Our approach is to acquire tissue from subjects undergoing surgery to remove pulmonary neoplasms and obtain an airway segment away from the tumour mass. As expected from a population pool undergoing surgery to remove pulmonary neoplasms, approximately half our subjects had a history of smoking. Former smokers had refrained from smoking cigarettes for at least eight years. While there was a smoking history in both the asthma and control groups, smoking may alter airway behaviour in vitro and influence comparisons between groups. Age and gender are uncontrolled variables. Most subjects were older than 60 years of age with one 20 year old in the history of asthma group and two subjects from the control group who were 39 and 43 years respectively. There was a notable gender bias with mostly female subjects in the history of asthma group and males in the control group. Control subjects did not have airflow obstruction, defined as an FEV$_1$/FVC ratio less than 0.7 and FEV$_1$ less than 80% predicted. Access to airway tissue from asthmatic subjects is especially difficult since asthmatic subjects make up only a fraction of the patients undergoing lung surgery, matching the prevalence of asthma in the general population (~15%).

Asthma was defined as a history of doctor-diagnosed asthma at any stage of life. All subjects thus defined were currently on asthma medication. Lung function was not different between the groups. The lack of a difference in lung function between groups could be due to the high variance in the data as a result of uncontrolled variables (smoking, age and gender) and a function of low statistical power. Only one subject from the history of asthma group responded to bronchodilator although subjects were not asked to withhold medication at the time of study. Treatment with steroids and long-acting beta agonists may have impacted our in vitro responses but cannot explain increased airway narrowing in the asthmatic group and should instead favour reduced narrowing. The one control subject who was on anticholinergic medication for a non-respiratory related disorder had no evidence that airway narrowing was affected and was above the mean for the control group.
The major advance of the *in vitro* technique used in this chapter over earlier studies is that we were able to measure the functional response of the airway wall (narrowing) during ASM shortening in the presence of normal physiological loads. The airway narrowing response in bronchial segments is more functionally related to bronchoconstriction measured in subjects *in vivo*, as opposed to measurements of ASM force or stress in isolated ASM strips or ring.

Airways from the asthmatic group had increased maximal airway narrowing to ACh *in vitro* and this was related to the area of the ASM layer, which equates to greater ASM mass since the fraction of ASM within the layer was similar between groups. A correlation between ASM mass and airway narrowing in airway disease has not previously been demonstrated to our knowledge despite often being assumed or hypothesised. Other structural and mechanical properties of the airway wall (e.g. inner/outer wall area and compliance and therefore load) were not related to airway narrowing. Predictions from mathematical models highlight the potential importance of the ASM in determining airway narrowing (Affonce and Lutchen 2006; Lambert, Wiggs *et al.* 1993) and suggest that increases in ASM mass identified in asthma is likely to underpin increased maximal airway narrowing. However these models make the assumption that with expansion of the ASM layer, force and shortening will also increase. Proliferation of ASM cells in culture may be associated with a less contractile ASM phenotype that would not favour increased airway narrowing (Chamley-Campbell, Campbell *et al.* 1979) and, theoretically, the thickened ASM layer may increase the internal loads (e.g. from more ECM) that would oppose shortening of ASM (Meiss 1999). Our present data show that the thickened ASM provides a mechanical advantage that can account for at least some of the increase in maximal airway narrowing observed in asthma and provide new physiological support for the mathematical models. Since the thickness of the inner airway wall remained unchanged (the ASM simply made up a greater fraction of the airway wall) it seems likely that airway narrowing was increased due to greater ASM force and shortening, rather than other geometric factors (Lambert, Wiggs *et al.* 1993).

Stereological measurements on the airways used in the *in vitro* studies were applied to determine the presence of ASM hypertrophy or hyperplasia. This thesis has previously demonstrated that hyperplasia rather than hypertrophy may be a feature of more severe
as asthma (Chapter 4) (James, Mauad et al. 2012) although hyperplasia has also been observed in milder cases (Woodruff, Dolganov et al. 2004). The present results showed no differences in the composition of the ASM layer between airways from control and history of asthma subjects. These negative results may be due to our sample size, which is low for such investigations. Additionally, while there were no differences in average airways size between groups, there was a range of Pbm in both groups which further increases the variance in the data. Therefore we cannot be certain if hyperplasia or hypertrophy of ASM cells contributed to the increased airway narrowing observed in the asthmatic airways in the present study.

Whether the contractile phenotype of ASM cells is altered in asthma is also not clear and there is evidence both for (Bai 1990; Bramley, Thomson et al. 1994) and against (Goldie, Spina et al. 1986; Whicker, Armour et al. 1988; Chin, Bosse et al. 2012) increased contractility. If there is a change in ASM contractile phenotype in asthma we would expect the relationship between the amount of ASM and narrowing to be different for asthmatic and control subjects. Our data do not suggest a change in the ASM phenotype as the relationship between amount of ASM and the degree of airway narrowing was the same in asthmatic and control airways.

It is now well established that the bronchodilatory response to DI is reduced in subjects with asthma (Brusasco, Crimi et al. 1999; Crimi, Pellegrino et al. 2002; Salome, Thorpe et al. 2003). The present study aimed to provide some insight as to whether an abnormality of the airway wall contributes to the dysfunction observed in vivo. We observed bronchodilatory responses to DI in subjects with a history of asthma, however these tended to be less than in the control group. One possible explanation may be that the observed increase in airway narrowing in the asthmatic group limits DI-induced bronchodilation. We also showed that when the level of airway narrowing (to carbachol) was matched, bronchodilation to DI was similar in airways from the control and asthmatic groups. This suggests that in asthma the airway wall itself retains a ‘normal’ response to mechanical stretch/stress which occurs during DI. These data dispute the theory that an abnormal mechanical response of the airway is the cause of the reduced bronchodilator response to DI seen in asthmatic patients. Regardless of the reason for the impaired response to DI in asthmatic subjects, the present study argues that failure in the response to DI is not the cause of airway hyperresponsiveness. The
airways from asthmatic subjects were more responsive to contractile agonists but retained a normal response to DI.

Although airway remodelling in asthma could theoretically stiffen the airway wall, we observed no changes in the specific compliance or pressure to collapse airways from asthmatic compared with non-asthmatic group. This suggests that the elastic properties of the wall are not altered in asthma. Lack of statistical power may be an issue, although there were no trends to suggest a difference in airway compliance between the groups. Given that there was no general airway remodelling other than an increase in the area of the ASM layer, this alone may not be sufficient to alter the compliance of the airway wall. Tiddens et al. (Tiddens, Hofhuis et al. 1999) also studied airways from subjects undergoing lobar resection, although these airways were smaller than those in the present study. They showed that prior to ASM stimulation the areas of the total and outer wall, but not the ASM layer, were inversely related to airway compliance. There was however an inverse relationship between the area of the ASM layer and compliance after stimulation to methacholine. Importantly, while several studies in vivo report reduced distensibility of airways in asthma (Brackel, Pedersen et al. 2000; Johns, Wilson et al. 2000) this does not necessarily imply that the airway wall itself is stiffer since the pressure-volume response of the airway is impacted by the interdependence between the airway and lung parenchyma. Airway-parenchymal uncoupling has been proposed as a mechanism modifying airway mechanics and may arise due to outer wall thickening or peribronchial oedema both of which would reduce the transmission of force to the airway (Macklem 1991).

This study provides the first data in human airways to demonstrate a correlation between ASM mass and airway narrowing, which implicates ASM in the pathogenesis of airway hyperresponsiveness. Consistent with this theory is that airways from subjects with a prior diagnosis of asthma had a thickened ASM layer and narrowed more. In comparison, airways from the history of asthma group retain a normal response to the mechanical stresses and strains accompanying tidal and DI oscillations. These findings suggest that airway hyperresponsiveness and the impairment in the bronchodilatory response to DI are unrelated since at the airway level, increased airway narrowing is demonstrated without any defect in the airway wall response to DI.
6.1 Introduction

This thesis has established that in asthma the increased thickness of the ASM layer is due to hyperplasia of ASM cells in the large, medium and small airways in fatal cases of asthma, and to hypertrophy of ASM cells in the large airways in cases of both fatal and non-fatal asthma, with no change to the relative fractions of ASM and ECM within the ASM layer (Chapter 4). The area of the ASM layer is also increased in severe cases of COPD (Hogg, Chu et al. 2004) however it is unknown if this is due to the same remodelling mechanisms that occur in asthma. As discussed, an increase in the thickness or area of the ASM layer may result from an increase in the size or number of ASM cells, and/or an increase in the volume of ECM or other elements within the ASM layer. These changes may also result in changes in the volume fractions of various tissue elements within the ASM layer. Only one previous study has examined ASM cell number and volume in subjects with COPD (Ebina, Yaegashi et al. 1990). These cases (n = 13) had mild airflow obstruction and were reported to have a mild degree of hypertrophy in the large airways, with the small airways remaining within the range of the controls. The amount of ECM within the ASM layer may have contributed to the apparent increase in ASM cell volume observed as it was not taken into account. There are no studies that have examined structural changes in the ASM layer across a range of COPD severities.

Studies that have examined ECM changes in COPD are also few, with the severity of COPD not always well defined. These studies show changes in the ECM in the airways of COPD subjects to be related to lung function (Black, Ching et al. 2008) suggesting that remodelling of the ECM could determine disease severity. Decreased elastin fibers (Black, Ching et al. 2008) and increased expression of collagens I, III, and IV, fibronectin and laminin (Kranenburg, Willems-Widyastuti et al. 2006) have all been observed in the airway wall of COPD subjects but not in the ASM layer itself. Quantification of ECM within the ASM layer has also only been performed in two studies. One study reported an increased staining intensity of laminin in COPD with no changes in the expression of other ECM proteins (Kranenburg, Willems-Widyastuti et
al. 2006). A recent study of COPD has shown the expression of collagen to be
decreased in the large and small airways, with tenascin and fibronectin expression both
increased (Annoni, Lancas et al. 2012). The study also examined the relative areas of
ECM, which is a major advance on previous studies. However, the ECM area was
assessed on relatively ‘thick’ (4µm) sections and would be subject to overlap (as
demonstrated in Chapter 3) which may have confounded the results. Due to the
potential importance of the ECM on lung function and disease severity in COPD, the
changes in the ECM matrix should be more extensively characterised and quantified.
Quantification of the ECM within the layer of the ASM was a primary focus of this
study.

This chapter examines ASM hypertrophy and hyperplasia, and the contribution of the
ECM in large and small airways of subjects with different severities of COPD. The aim
of this study was to determine the relative contributions of remodelling of ASM and
ECM to the ASM layer in subjects with COPD. We hypothesised that the increased
ASM layer in COPD subjects is due to an increase in the volume fraction of ECM
within the smooth muscle layer, with no change in the size or number in ASM cells.

6.2 Methods

6.2.1 Subjects
Post-mortem and post-operative tissues of large and small airways were used for this
study. Post-operative tissues (n = 8) were collected from the laboratory of Professor
James Hogg in Vancouver, Canada. Post-operative tissues (n = 15) were collected from
Sir Charles Gairdner hospital in Perth, Western Australia, from subjects undergoing
lung resection surgery. Post-mortem tissues from the Perth tissue bank were also used
(n = 7).

Values for spirometry (pre- and post-bronchodilator) were obtained from hospital
records for post-mortem cases and spirometry was performed within a week prior to
surgery for the post-operative cases. Given the various sources of the tissue, spirometry
was performed on a number of different devices, but always to American Thoracic
Society standards (1995). Values for FEV₁ and FVC were used to characterise subjects
by GOLD stage I – IV using the 2012 classification (Section 2.1) (Decramer 2012).
Normal lung function is defined as a post-bronchodilator FEV₁/FVC >0.7 and FEV₁% >80% predicted.

All tissue was fixed in formalin and processed as specified in the methods (Section 2.4). Subjects were categorised as non smoking controls (never smoked), smoking controls (current or ex-smokers with normal lung function), mild COPD (GOLD I and GOLD II), severe COPD (GOLD III and GOLD IV) based on lung function data. The number of subjects in each group, demographics and lung function are presented in tables 6.1 and 6.2.

6.2.2 Structural Assessment
For this study we have used the optical disector approach (Section 2.7) to determine ASM cell size and number on 30μm sections, and 0.5μm sections to estimate the volume fractions of ASM and ECM (Section 2.6).

6.2.3 Statistics
Data were grouped by airway size defined as small airways with a Pbm <6mm, and large airways with a Pbm >6mm. The volume fraction of ASM, ECM and Other, and Nᵥ, Vᵥ and Nᵥ were assessed in small and large airways using a One Way ANOVA and appropriate post-hoc tests (Holm-Sidak or Kruskal-Wallia as appropriate). Correlation analysis were performed for all ASM parameters against FEV₁% predicted, FEV₁/FVC and pack years.

6.3 Results

6.3.1 Subject Characteristics
Subject characteristics are shown in Tables 6.1 and 6.2. Age and gender were uncontrolled random variables. COPD subjects included similar numbers of current and ex-smokers. The severe COPD group showed a significantly greater history of smoking (pack years) compared with controls.
6.3.2 Airways Size and ASM/Pbm
Airway size, assessed by Pbm, was similar in all groups except in GOLD III where Pbm was lower. This difference was significant when compared with the non-smoking control group when all airway sizes were examined (p <0.05) (Figure 6.1). To reduce the impact of an airway size bias, airways were analysed separately after being split into small (Pbm <6mm) and large (Pbm >6mm) airways. No differences in Pbm were seen between GOLD stages when airways were stratified into small (p = 0.28) and large airway groups (p = 0.15). The thickness of the ASM layer (√ASM/Pbm) for all airways, in each subject group is plotted in Figure 6.2. No differences were seen in √ASM/Pbm between GOLD stages (p = 0.33). Similarly there were no significant differences between groups when small (p = 0.49) or large airways (p = 0.32) were analysed separately.

Table 6.1: Subject characteristics

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Gender</th>
<th>Age yrs</th>
<th>Height cm</th>
<th>Weight kg</th>
<th>Smoking</th>
<th>Pack years</th>
</tr>
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<tr>
<td>Non-smoking Controls</td>
<td>M / F</td>
<td>54 ± 13</td>
<td>166 ± 9</td>
<td>72 ± 17</td>
<td>0 / 6 / 0 / 0 / 0</td>
<td>0</td>
</tr>
<tr>
<td>Smoking Controls</td>
<td>4 / 2</td>
<td>64 ± 10</td>
<td>168 ± 12</td>
<td>81 ± 12</td>
<td>5 / 0 / 1 / 0 / 0</td>
<td>39 ± 27</td>
</tr>
<tr>
<td>Mild COPD</td>
<td>7 / 3</td>
<td>71 ± 5</td>
<td>172 ± 11</td>
<td>78 ± 15</td>
<td>3 / 0 / 7 / 0 / 0</td>
<td>35 ± 30</td>
</tr>
<tr>
<td>Severe COPD</td>
<td>3 / 7</td>
<td>62 ± 5</td>
<td>165 ± 5</td>
<td>72 ± 21</td>
<td>3 / 0 / 5 / 2 / 0</td>
<td>58 ± 39*</td>
</tr>
</tbody>
</table>

* p<0.05 compared with non-smoking controls; # mean ± SD

Table 6.2: Subject lung function

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Pre-Bronchodilator</th>
<th>Post-Bronchodilator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV₁ (% pred)</td>
<td>FVC (% pred)</td>
</tr>
<tr>
<td>Non Smoking Controls</td>
<td>99.0 ± 11.5</td>
<td>97.4 ± 11.0</td>
</tr>
<tr>
<td>Smoking Controls</td>
<td>83.5 ± 17.8</td>
<td>82.4 ± 15.3</td>
</tr>
<tr>
<td>Mild COPD</td>
<td>75.5 ± 14.6</td>
<td>89.8 ± 16.1</td>
</tr>
<tr>
<td>Severe COPD</td>
<td>30.4 ± 14.8</td>
<td>54.7 ± 13.6</td>
</tr>
</tbody>
</table>
When GOLD stages were combined into mild and severe COPD groups, there were no significant differences observed in the thickness of the ASM layer (p = 0.23, Figure 6.3).

Figure 6.1: Size (Pbm) of all airways examined in non-smoking control (NSC) and smoking control (SC) groups, and GOLD stages I – IV. (* p <0.05)

Figure 6.2: ASM area relative to airway size (√ASM/Pbm) of all cases in non-smoking control (NSC) and smoking control (SC) groups, and GOLD stages I – IV. Each point represents the mean of large and small airways for each subject.
Figure 6.3: ASM area by airway size (\(\sqrt{\text{ASM/Pbm}}\)) of all cases when grouped into non-smoking (NSC) and smoking control (SC) groups, and mild and severe COPD groups.

6.3.3 Volume Fractions of the ASM Layer - ASM, ECM and "Other"

\(V_{\text{VASM}}\) was not different between groups in either small (\(p = 0.30\)) or large airways (\(p = 0.60\), Figure 6.4A). In the large airways, \(V_{\text{VECM}}\) was increased in severe COPD compared with control groups and mild COPD (Figure 6.4B). In airways where \(V_{\text{VECM}}\) was increased there was a corresponding decrease in \(V_{\text{VOther}}\). \(V_{\text{VOther}}\) was reduced in the severe COPD compared with mild COPD in both the small and larger airways (Figure 6.4C). There were no significant differences between groups for \(V_{\text{VECM}}\) in the small airways (\(p = 0.093\)).
Figure 6.4: \( V_{\text{VASM}} \) (A), \( V_{\text{VECM}} \) (B) and \( V_{\text{VOther}} \) (C) in small and large airways in non-smoking controls, smoking controls, mild COPD and severe COPD groups. (* \( p < 0.05 \))

6.3.4 Numerical Density (\( N_V \)), Mean Volume of ASM cells (\( V_C \)) and Number of ASM Cells per Unit Length of Airway (\( N_L \))

There were no differences in \( N_V \), \( V_C \) or \( N_L \) between groups in the large or small airways (Figure 6.5). \( N_L \) was increased in large compared with small airways (\( p < 0.01 \)).
Figure 6.5: Numerical volume density ($N_V$) (A) and mean volume ($V_C$) (B) of ASM cells, and the number of ASM cells per unit length of airway ($N_L$) (C) in small and large airways in non smoking controls, smoking controls, mild and severe COPD groups.

6.3.5 Relationship of Airway Structure to FEV$_1$% Predicted and FEV$_1$/FVC

$V_{VEC}$ was negatively correlated with lung function (FEV$_1$ % predicted and FEV$_1$/FVC) in the large (Figure 6.6) but not the small airways (FEV$_1$ % predicted, $p = 0.08$; FEV$_1$/FVC $p = 0.71$). $V_{Other}$ was positively correlated with FEV$_1$% predicted and FEV$_1$/FVC in the small (Figure 6.7) and large airways (Figure 6.8). $N_V$ inversely correlated with FEV$_1$/FVC in small and large airways (Figure 6.9) and FEV$_1$% predicted fell just below the level of significance in the large airways (Figure 6.10). FEV$_1$% predicted was not related to $N_V$ in the small airways ($p = 0.13$). Neither FEV$_1$
(% pred) or FEV<sub>1</sub>/FVC were related to V<sub>VASM</sub>, V<sub>C</sub> or N<sub>L</sub> in small or large airways (Table 6.3).

Figure 6.6: Relationship between lung function (A, FEV<sub>1</sub>% predicted; B, FEV<sub>1</sub>/FVC) and V<sub>VECM</sub> in the large airways.

Figure 6.7: Relationship between lung function (A, FEV<sub>1</sub>% predicted; B, FEV<sub>1</sub>/FVC) and V<sub>VOther</sub> in the small airways.
Figure 6.8: Relationship between lung function (A, FEV₁% predicted; B, FEV₁/FVC) and \( V_{V_{Other}} \) in the large airways.
Figure 6.9: Relationship between lung function (FEV₁/FVC) and numerical density of ASM cells (NV) in small (A) and large (B) airways.

Figure 6.10: Relationship between lung function (FEV₁% predicted) and numerical density of ASM cells (NV).
Table 6.3: Relationships between lung function (FEV₁% pred and FEV₁/FVC) and measured ASM parameters.

<table>
<thead>
<tr>
<th>ASM parameter</th>
<th>FEV₁ % Predicted</th>
<th>FEV₁/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small airways</td>
<td>Large airways</td>
</tr>
<tr>
<td>( V_{VASM} )</td>
<td>p = 0.66</td>
<td>p = 0.30</td>
</tr>
<tr>
<td></td>
<td>r = -0.085</td>
<td>r = -0.208</td>
</tr>
<tr>
<td>( V_C (\mu m^3) )</td>
<td>p = 0.78</td>
<td>p = 0.16</td>
</tr>
<tr>
<td></td>
<td>r = 0.054</td>
<td>r = 0.269</td>
</tr>
<tr>
<td>( N_L ) (cells/mm)</td>
<td>p = 0.08</td>
<td>p = 0.36</td>
</tr>
<tr>
<td></td>
<td>r = 0.327</td>
<td>r = 0.182</td>
</tr>
</tbody>
</table>

*p and r values presented for analysis in small and large airways. Abbreviations: \( V_{VASM} \) = volume fraction of ASM; \( V_C \) = mean volume of ASM cells; \( N_L \) = the number of ASM cells per unit length of airway.

### 6.3.6 Effects of Smoking

As smoking cigarettes is globally the major risk factor for the development of COPD, the relationship between the number of years smoked (pack-years) and components of the ASM layer was assessed. In large airways, \( V_{VECM} \) was positively related to pack-years (Figure 6.10A), while \( V_{VOther} \) decreased with the greater pack-years (Figure 6.10B). There was no relationship between pack-years and \( V_{VASM} \), \( N_V \), \( V_C \) or \( N_L \) in the small or large airways (Table 6.4).

![Figure 6.10: Relationship between ASM layer components (\( V_{VECM}, A; V_{VOther}, B \)) and smoking (Pack year = number of years smoked x number of packs per day).](image-url)
Table 6.4: Relationship between smoking (pack years) and ASM parameters in large and small airways (p and r values presented).

<table>
<thead>
<tr>
<th>ASM parameter</th>
<th>Small Airways</th>
<th>Large Airways</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{VASM}$</td>
<td>$p = 0.73$</td>
<td>$p = 0.84$</td>
</tr>
<tr>
<td></td>
<td>$r = -0.068$</td>
<td>$r = 0.042$</td>
</tr>
<tr>
<td>$N_v (\text{cells/mm}^3)$</td>
<td>$p = 0.95$</td>
<td>$p = 0.70$</td>
</tr>
<tr>
<td></td>
<td>$r = 0.011$</td>
<td>$r = 0.079$</td>
</tr>
<tr>
<td>$V_c (\mu m^3)$</td>
<td>$p = 0.75$</td>
<td>$p = 0.63$</td>
</tr>
<tr>
<td></td>
<td>$r = -0.061$</td>
<td>$r = -0.098$</td>
</tr>
<tr>
<td>$N_L (\text{cells/mm})$</td>
<td>$p = 0.20$</td>
<td>$p = 0.25$</td>
</tr>
<tr>
<td></td>
<td>$r = -0.247$</td>
<td>$r = -0.232$</td>
</tr>
</tbody>
</table>

6.4 Discussion

The work described in this chapter is the first to quantify the size and number of ASM cells as well as the fractional component of ECM within the ASM layer in airways from subjects with mild to severe COPD. Although an increase in the thickness of the ASM layer was not observed in subjects with COPD the study demonstrated that in COPD there is an increased fraction of ECM within the ASM layer, with no changes to the ASM cell size or number. The volume fraction of ECM was inversely related to lung function and increased with pack-years of smoking. The changes to the ASM layer in COPD appear distinctly different from those seen in asthma (Chapter 4), and likely reflect different underlying mechanisms of disease.

Previous studies have shown that the ECM in airways from subjects with COPD has increased expression of specific proteins, associated with more severe disease (Baraldo, Bazzan et al. 2007; Kranenburg, Willems-Widyastuti et al. 2006), consistent with our findings. In the present study, since the fraction of ECM was increased in the ASM layer of airways from subjects in the severe COPD group, while the area of the layer was unchanged, the amount (absolute) of ECM was therefore increased. That this study did not observe an increase in the area of ASM in COPD subjects, as previously been described (Hogg, Chu et al. 2004; Kuwano, Bosken et al. 1993; Saetta, Di Stefano et al. 1996).
1998), may be due to the relatively low sample size (n = 20 with COPD) which has been gathered from two different sources (post-mortem and post-operatively from Canada and Australia). The group with severe COPD had greater volume fraction of ECM compared with the mild COPD group and non-smoking controls. There was also a correlation between the volume fraction of ECM and reduced lung function (FEV$_1$% predicted and FEV$_1$/FVC). This reflects results by Kranenburg et al. (Kranenburg, Willems-Widyastuti et al. 2006) who observed an inverse relationship between FEV$_1$% predicted and the intensity of laminin staining in the ASM layer in subjects with COPD. It is possible that other ECM proteins may also have increased expression in the ASM layer in COPD, however few studies have addressed this possibility. These results suggest that fixed airflow limitation involves remodelling of the ECM, which may restrict movement of the ASM and reduce the capacity for the airway to dilate.

An increase in ECM within the ASM layer was associated with a corresponding reduction in the volume fraction of ‘other’. The fraction of ‘other’ was almost 50% greater in the mild COPD group, while the fraction of ASM was unchanged. ‘Other’ was scored in the point counting method when points fell on space between ASM cells and matrix, or on the occasional blood vessels and inflammatory cells. That ‘other’ was reduced in airways from subjects with COPD can be explained by the deposition of matrix within this space such that the volume fraction of ECM increases and of ‘other’ decreases. It is possible that ‘other’ also represents elements of the ASM layer that are unstained by the Masson’s Trichrome technique. Although this technique is known to stain ASM cells red and ECM blue, it may be that this stain does not stain all ECM proteins. That the volume fraction of ‘other’ was somewhat higher in the mild COPD group (though not statistically different from controls) may represent ECM fibres that were not revealed by the stains employed. The implication is that that the type of ECM proteins may differ in severe COPD compared with milder forms of the disease.

As smoking is also a well-known factor in the development of COPD (Buist, McBurnie et al. 2007) we examined the relationship between cigarette smoking (pack-years) and the amount of ECM. We have found that $V_{VECM}$ increased in accordance with the number of pack-years smoked. This relation to pack-years suggests that the $V_{VECM}$ is related to a decline in lung function, not just to starting lung function, as cigarette smoke exposure is one of the few things that is strongly related to lung function decline.
(James, Palmer et al. 2005). This relationship to pack-years compares well with a study by Krimmer (Krimmer, Burgess et al. 2012) who showed cigarette-exposed lung fibroblasts from subjects with COPD had increased deposition of fibronectin compared with non-cigarette exposed tissue. Fibroblasts were incubated with 0.5% and 5% cigarette smoke, with fibronectin deposition increased in both instances but only significant at the higher smoke exposure level. No changes in the deposition of laminin, elastin, tenascin, collagen or versican were observed in either smoke-exposure level. When fibroblasts from subjects without airflow obstruction were exposed to cigarette smoke, no change in ECM expression was observed although the expression of fibronectin was slightly increased. These results suggest that smoking may play an important role in the increased expression of ECM within the ASM layer and through this mechanism, may give rise to COPD.

When the number and size of the ASM cells were examined, no change was found between control groups and cases of COPD. There was a trend for decreased N_L in the large airways for the severe group of COPD compared with smoking controls, however this did not reach significance (p = 0.087). This trend could be explored further with increased numbers severe cases of COPD (n = 13 in the present study). Cigarette smoking was not found to be related to ASM cell size or number, however N_V was related to lung function. An increased N_V within the ASM layer volume was associated with a decrease in the FEV_1/FVC ratio. In the presence of a greater volume fraction of ECM, the ASM may be fixed within a rigid sheath that could prevent further shortening and also dilation (Mijailovich, Fredberg et al. 1996), reducing the effectiveness of bronchodilator therapy in this patient group.

Taking into consideration the findings of this study, we consider the role of cigarette smoking in the development of COPD. Smoking (pack-years) positively correlated with greater volume fraction of ECM within the layer of ASM and negatively correlated with FEV_1 (% predicted) (p = 0.025). The consequence of inhaled cigarette smoke may be an increase in the amount of ECM or a shift in the type of ECM that is normally expressed in the airway. The functional consequence of increased ECM may be a stiff airway wall that, as discussed, does not respond well to constrictor or dilator stimuli. In Chapter 7 we test the hypothesis that smoking-induced airway disease stiffens the airway wall and reduces the airway narrowing to broncho-constrictor stimuli.
CHAPTER 7: Effects of Smoking on Airway Smooth Muscle Structure and Mechanics

7.1 Introduction

There are structural differences in the lung parenchyma and airways between asthma and COPD which suggest that the underlying disease mechanisms differ. In Chapter 4 it was shown that the airways of subjects with asthma exhibit hypertrophy and hyperplasia of the ASM, in contrast to the airways in subjects with COPD which show no changes in ASM cell number or size, but instead, an increase in the volume fraction of ECM. These differences in airway remodelling may explain apparent differences in the functional behaviour of subjects with asthma and COPD. Patients with asthma typically show a loss of the normal limit to airway narrowing (i.e. increased maximal airway narrowing), however respond well to bronchodilator therapy. In comparison, patients with COPD have smoking-related inflammation and remodelling, regularly demonstrate a plateau in the bronchoconstrictor response during conventional challenge (Figure 7.1A) and respond poorly to inhaled bronchodilators (Celli and MacNee 2004; Rabe, Hurd et al. 2007).

Typically when measuring airway responsiveness, the response to bronchodilator is measured as a percentage change from baseline lung function. As COPD subjects usually start with a lower baseline lung function, their response will predictably be less. When change in FEV₁ is shown in millimetres rather than as a percent of baseline (Figure 7.1B), the absolute change in lung function in COPD is seen to be very small, even less than these seen in subjects with normal lung function. This suggests that only a small amount of smooth muscle shortening occurs in these airways when bronchoconstrictor is administered, possibly due to existing airway narrowing that is present due to remodelling (Lambert, Wiggs et al. 1993). The airways in COPD are therefore both difficult to ‘open’ and difficult to ‘close’ suggesting a pathology that restricts ASM movement.
We propose that changes to the ECM within the ASM layer accounts for different behaviours of the airway wall in COPD and asthma, and that these changes are induced by smoking. Increased volume fraction of ECM in the ASM layer is inversely related to FEV₁, and the amount of ECM increases in accordance with the number of pack-years smoked (Chapter 6). We hypothesise that an increased volume fraction of ECM within the ASM layer is associated with increased stiffness of the ASM layer, restricting movement to both contractile and relaxant stimuli. The aim of the present study was to examine the relationship between cigarette smoking, airway mechanical properties and structure of the ASM layer in human bronchi.

### 7.2 Methods

#### 7.2.1 Subjects

Subjects were recruited from Sir Charles Gairdner Hospital and the Mount Hospital within ten days prior to lobectomy or pneumonectomy for the removal of pulmonary neoplasms. All patients performed tests of standard lung function and were administered a questionnaire on smoking history and respiratory illness and symptoms and had skin prick tests to common aero-allergens (Section 2.3). Pack years of smoking (Section 2.8.4) was calculated for all subjects.
7.2.2 *Experimental Protocol*
Bronchial segments were obtained from the resected lung specimen, after dissection for pathological staging by the attending pathologist. Isolated bronchial airway segments were mounted in a custom-made organ bath system for measurement of airway mechanics (Section 2.9). Broncho-constriction (reduction in lumen volume, %) to acetylcholine, airway compliance (cmH₂O⁻¹), pressure to collapse (airway closing pressure, cmH₂O) and response to theophylline (increase in lumen volume, %) were measured. Airways were subsequently fixed in formaldehyde for structural assessment.

7.2.3 *Structural Assessment*
To assess airway structure, consecutive transverse sections of airway segments were cut at 0.5µm, 4µm and 30µm thickness. The area of the ASM layer (AASM), ASM cell volume density (NV) and mean ASM cell volume (VC) and the number of ASM cells per unit length of airway (NL) were estimated on 30µm sections (haematoxylin) using stereological techniques (Section 2.7). The perimeter of the basement membrane, inner wall area, outer wall area and ASM area were measured on 4µm sections stained with haematoxylin and eosin. Thin 0.5µm sections were used to estimate the volume fractions (VV) of ASM cells and ECM using the point counting technique (Section 2.6).

7.2.4 *Statistics*
Pack-years of smoking were correlated with mechanical airway properties (e.g. compliance), airway function (e.g. airway narrowing) and morphological measurements using Pearson’s correlation analysis.

7.3 *Results*

7.3.1 *Subject Characteristics*
Subject characteristics are presented in Table 7.1. Subjects had no history of asthma and were all current (n = 6) or ex-smokers (n = 6) with an average age of 66 ± 6 years with a smoking history of 45 ± 30 pack-years. *In vitro* airway segment length (17.1 ± 5.4mm) and airway size (11.1 ± 3.8mm) for each subject are listed in Table 7.2.
Table 7.1: Subject characteristics

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Gender</th>
<th>Age yrs</th>
<th>FEV₁ (% pred)</th>
<th>FVC (% pred)</th>
<th>FEV₁/FVC</th>
<th>Smoking</th>
<th>Pack years</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>66</td>
<td>66.74</td>
<td>63.80</td>
<td>0.78</td>
<td>Ex</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>66</td>
<td>67.65</td>
<td>84.46</td>
<td>0.60</td>
<td>Y</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
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<td>52.55</td>
<td>60.43</td>
<td>0.65</td>
<td>Y</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
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<td>72.10</td>
<td>69.50</td>
<td>0.78</td>
<td>Ex</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>50</td>
<td>83.54</td>
<td>92.79</td>
<td>0.70</td>
<td>Y</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>73</td>
<td>59.05</td>
<td>63.84</td>
<td>0.68</td>
<td>Ex</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>72</td>
<td>88.79</td>
<td>99.53</td>
<td>0.65</td>
<td>Ex</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>70</td>
<td>56.62</td>
<td>61.62</td>
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<td>Ex</td>
<td>16</td>
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<td>9</td>
<td>M</td>
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<td>90.40</td>
<td>91.98</td>
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<td>Ex</td>
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<tr>
<td>11</td>
<td>F</td>
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<td>12</td>
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<td>73.47</td>
<td>91.14</td>
<td>0.62</td>
<td>Y</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 7.2: Airway length and size (Pbm) of in vitro preparations.

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Airway length mm</th>
<th>Airway size Pbm, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.5</td>
<td>12.0</td>
</tr>
<tr>
<td>2</td>
<td>23.6</td>
<td>13.0</td>
</tr>
<tr>
<td>3</td>
<td>25.9</td>
<td>18.2</td>
</tr>
<tr>
<td>4</td>
<td>19.1</td>
<td>12.6</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>16.1</td>
</tr>
<tr>
<td>6</td>
<td>21.2</td>
<td>11.9</td>
</tr>
<tr>
<td>7</td>
<td>18.2</td>
<td>7.0</td>
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<tr>
<td>8</td>
<td>17.0</td>
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<td>10</td>
<td>18.0</td>
<td>11.5</td>
</tr>
<tr>
<td>11</td>
<td>7.6</td>
<td>5.6</td>
</tr>
<tr>
<td>12</td>
<td>14.3</td>
<td>6.6</td>
</tr>
</tbody>
</table>
7.3.2 Functional Relationships

Cigarette smoking (pack-years smoked) was inversely correlated with airway compliance and the airway closing pressure (Figure 7.2A, B). That is, airways from subjects who had a greater pack-year history were less compliant and required a more negative (greater compressive pressure) to bring them to collapse. There was also a trend towards reduced airway narrowing with greater pack-years of smoking, but this did not reach significance (p = 0.09).

Response to theophylline was assessed with airways in their relaxed state, that is without contraction to exogenous stimuli. Airway response to theophylline was stratified into two different groups, those that did respond (>15% relaxation – “responders”) and those that did not (<5% relaxation – “non responders”). Non-responders tended to smoke more although there was considerable overlap between the groups (Figure 7.3).

Figure 7.2: Cigarette smoke exposure (pack-years) was inversely related to airway closing pressure (A) and airway compliance (B).
Figure 7.3: Pack-years of smoking (mean ± standard deviation) for subjects whose airways showed <5% relaxation (non-responders) or >15% relaxation (responders) to theophylline in vitro.

7.3.3 Structural Relationships

Pack-years of smoking was positively related to $V_{VECM}$ within the ASM layer (Figure 7.4A), and tended to be negatively related to $V_{VASM}$ ($p = 0.06$) (Figure 7.4B). There was no significant relationship between pack-years of smoking and $V_{Vother}$ ($p = 0.26$). There was also no relationship between pack-years of smoking and $V_{V}$ ($p = 0.60$), $V_C$ ($p = 0.94$), $N_L$ ($p = 0.89$), $A_{ASM}$ ($p = 0.47$) or areas of the inner ($p = 0.26$) or the outer airway walls ($p = 0.30$).

Figure 7.4: Volume fractions of airway smooth muscle, $V_{VASM}$, (A) and extracellular matrix, $V_{VECM}$ (B) within the ASM layer, in relation to pack-years of smoking.
7.3.4 Relationship between Structure and Function

Airway closing pressure was positively related to $V_{VASM}$ ($p < 0.05$), and tended to be negatively correlated to $V_{VECM}$ ($p = 0.07$) (Figure 7.5A, B). That is, the more $V_{VASM}$, the less negative the pressure required to close the airways (the easier they were to collapse), and the more $V_{VECM}$, the more negative the pressure required to close the airways (the harder they were to collapse). Airway closing pressure was not significantly related to $V_{VOther}$ ($p = 0.6$), to the ASM volume density ($p = 0.17$), average ASM cell volume ($p = 0.4$) or number of ASM cells per unit length of airway ($p = 0.9$).

There were no significant relationships between airway compliance and the volume fraction of ASM, ECM or “other”.

There were no significant relationships between the in vitro response to theophylline and airway structural parameters ($V_{VASM}$, $V_{VECM}$, $N_V$, $V_C$, $N_L$). Non-responders showed a trend for increased $V_{VECM}$ ($p = 0.1$) compared with responders (Figure 7.5).

![Figure 7.5: Airways closing pressure in relation to the volume fraction of ASM ($V_{VASM}$, A) and the volume fraction of ECM ($V_{VECM}$, B) in isolated airway segments from smokers.](image-url)
Figure 7.6: Volume fractions of extracellular matrix, $V_{VECM}$ (mean ± standard deviation) within the ASM layer in subjects whose airways showed <5% relaxation (Non-Responders) or >15% relaxation (Responders) to theophylline in vitro.

7.3.5 Relationship of Airway Structure and Function to Airway Size

There were no significant relationships between airway size and any functional or structural measures (Table 7.3) although larger airways tended to be from more heavy smokers ($p = 0.056$) and tended to be negatively related to airway narrowing ($p = 0.08$).

Table 7.3: Relationship between airway size, determined by the perimeter of basement membrane (Pbm), and functional and structural parameters.

<table>
<thead>
<tr>
<th>Airway Parameter</th>
<th>p-value</th>
<th>r-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compliance (cmH$_2$O$^{-1}$)</td>
<td>0.53</td>
<td>0.21</td>
</tr>
<tr>
<td>Closing Pressure (cmH$_2$O)</td>
<td>0.41</td>
<td>0.27</td>
</tr>
<tr>
<td>Airway Narrowing (ΔV %)</td>
<td>0.31</td>
<td>-0.32</td>
</tr>
<tr>
<td>$V_{VASM}$</td>
<td>0.56</td>
<td>-0.20</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.73</td>
<td>0.12</td>
</tr>
<tr>
<td>$V_{VOther}$</td>
<td>0.55</td>
<td>0.20</td>
</tr>
<tr>
<td>$N_V$ (cells/mm$^3$)</td>
<td>0.96</td>
<td>-0.01</td>
</tr>
<tr>
<td>$V_C$ ($\mu$m$^3$)</td>
<td>0.96</td>
<td>-0.01</td>
</tr>
<tr>
<td>$N_L$ (cells/mm)</td>
<td>0.23</td>
<td>0.39</td>
</tr>
</tbody>
</table>
7.4 Discussion

In this chapter isolated airway segments were used to examine the relationship between cigarette smoking and the mechanical properties and structure of the ASM layer in human bronchi. We have shown cigarette smoking to be inversely related to airway compliance and the pressure required to collapse airways (the closing pressure). The closing pressure was also related to the volume fraction of ASM and pack-years of smoking was related to the volume fraction of ECM. No relationships were seen between airway compliance or closing pressure and the area of the ASM layer, the number of ASM cells and the average ASM cell volume. Taken together these results suggest that the composition of the ASM layer, rather than changes of the ASM cells themselves, is altered by smoking and that this favours wall stiffening.

The *in vitro* system used in this study was also used for our examination of airway narrowing and bronchodilation in asthma (Chapter 5), and as such previous limitations and advantages of this approach also apply here. Our rational for using this technique is that it provides a direct measure of airway wall mechanical properties separate from other complicating factors including the lung parenchyma. This is particularly important when studying the effects of cigarette smoke as it eliminates the confounding factor of general lung diseases (emphysema) which is present to varying degrees in most smokers (Patel, Coxson et al. 2008). However this technique requires sufficient tissue to mount a cylinder of airway in the organ bath, which limits sample size. Access to tissue is restricted to subjects undergoing lung resection surgery so gender and age of the subjects are uncontrolled variables. The age of subjects was similar (range 50 – 73 years) and the majority of subjects were male, possibly reflecting the previous smoking habits of this age group. Airway size is also to a degree an uncontrolled variable but by necessity all were cartilaginous bronchi (Pbm >6mm). The impact of airway size on the apparent relationship between smoking, ASM layer composition and airway wall mechanics is discussed later in this chapter.

A relationship between smoking (pack-years) and $V_{\text{VECM}}$ (airway structure) was previously shown in Chapter 7. In this chapter we have examined the relationship of cigarette smoking and airway mechanics to determine whether smoking could alter airway function. We hypothesised that an increased $V_{\text{VECM}}$ within the ASM layer results
from smoking cigarettes and causes increased stiffness of the airway wall. Airway compliance (change in airway lumen volume per change in lumen pressure) was measured during a small inflationary manoeuvre (comparable to that expected during tidal breathing) and the collapsing pressure was the compressive pressure required to close the airway. A more negative value for collapsing pressure indicates greater wall stiffness. Consistent with our hypothesis, more pack-years of smoking was associated with reduced airway compliance and a more negative closing pressure, indicating reduced collapsibility. Airway narrowing to acetylcholine also tended to be reduced with more pack-years of smoking (p=0.09), suggesting a limitation to ASM shortening.

We further examined the relationships between stiffness of the airway wall, pack-years of smoking and composition of the ASM layer. Our hypothesis was that airways with a greater $V_{\text{VECM}}$ in the ASM layer would be stiffer and resist narrowing. Airways with more ECM tended to have a more negative closing pressure ($p = 0.07$) indicating greater wall stiffness, and these airways also had a smaller $V_{\text{VASM}}$ in the ASM layer ($p <0.05$). These changes may prevent physical movement of the ASM layer, including dilatation in response to bronchodilators (see below). In comparison, there were no changes to the size or number of the ASM cells. This suggests that the increased $V_{\text{VECM}}$, rather than changes in the ASM, contribute to increased airway stiffness in smokers.

Responses of the airways to a bronchodilating agent were assessed at the end of the protocol when theophylline was added to identify intrinsic tone. When this was performed, the airway response to theophylline clearly fell into two categories, those that did respond (>15% relaxation “responders”) and those that did not (<5% relaxation “non-responders”). In line with our hypothesis, we questioned whether there were differences between the groups that could explain a reduced/increased response to theophylline. Results indicated that non-responders were airways which expressed a greater $V_{\text{VECM}}$ ($p = 0.15$) and whose subjects tended to have greater cigarette smoke exposure ($p = 0.1$). While these differences were not statistically different (possibly due to the small sample size), results are consistent with the notion that ASM in subjects with a greater pack-year history of smoking are less responsive to bronchodilator agents. This may be due to a greater volume fraction of $V_{\text{VECM}}$ within the ASM layer that restricts airway movement. The effects of smoking on airway structure and mechanics may therefore contribute to the functional differences observed between
COPD and asthma and explain the relative lack of an effect of bronchodilators (or bronchoconstrictors) observed in COPD subjects.

Due to the low sample size, there are potential confounders that require discussion. The major confounding factor for this study was airway size. Although all of the airways were, by our definition, “large” the range of airway sizes (Pbm 6 – 18mm) was quite wide. The larger airways also tended to be from more heavy smokers and tended to narrow less, which may have contributed to the relationships observed between smoking history and airway movement. The very large airways (Pbm >12mm) did show an increased $V_{VECM}$ ($p = 0.03$), which is consistent with our theory for increased airways wall stiffness. They also had a larger number of ASM cells per unit length of airway ($p = 0.02$). There were no other differences between the very large airways and the other airways examined. While these confounders could influence the conclusions drawn from the data, the collective body evidence generated from this thesis suggests that a shift in the composition of the ASM layer alters lung function, which may have a direct effect on airway wall mechanics. Larger studies are required to confirm independence of these findings from airway size.

In summary, this chapter implicates cigarette smoking as an important factor in the development of fixed airflow obstruction. Results suggest that fixed airflow limitation is strongly associated with cigarette smoking and involves remodelling of the ECM, which may restrict movement of the ASM (i.e. stiffen the ASM layer and airway wall). These findings have direct implications for how we approach the treatment of subjects with COPD, which is predominantly a smoking-related condition. COPD is often viewed as a condition similar to asthma because it is associated with airway narrowing, remodelling and inflammation. We propose instead that the underlying mechanisms in COPD are fundamentally different and may account for the fixed airway narrowing in COPD. Larger studies of the structure/function relationship in COPD and the effect of smoking should be undertaken to better understand the mechanisms involved in the development of COPD.
CHAPTER 8: Airway Response to Deep Inspiration in Asthma and COPD

8.1 Introduction

In normal subjects, taking a deep inspiration, (DI) induces a bronchodilator response and also prevents airway narrowing (broncho-protection) (Brusasco, Crimi et al. 1999; Skloot, Permutt et al. 1995). In diseases including asthma and COPD, responses to DI may be impaired, predisposing airways to excessive airway narrowing (Nadel and Tierney 1961; Ingram 1987; Burns, Taylor et al. 1985; Burns and Gibson 1998; Brusasco, Crimi et al. 1999; Crimi, Pellegrino et al. 2002). It is still not fully understood why the response to DI is altered in disease. A well-accepted hypothesis is that the reduced response to DI results from increased stiffness of the airway wall due to remodelling of the ASM and airway wall (Scichilone, Bruno et al. 2005; Skloot and Togias 2003). If remodelling is accompanied by an increase in airway stiffness, then the ASM is likely to be stretched less during a DI manoeuvre and any effect on ASM contraction is lessened (Fredberg 1998; Gunst, Shen et al. 2001; Noble, McFawn et al. 2007). The purpose of this chapter was to examine the relationship between the in vivo response to DI and the structure of the ASM layer.

8.2 Methods

8.2.1 Subjects

Subjects were recruited from Sir Charles Gairdner Hospital and the Mount Hospital several days prior to surgery for the removal of pulmonary neoplasms. Subjects completed a questionnaire on respiratory health, had an allergy skin prick test and performed a modified spirometry manoeuvre. The response to DI was assessed before and after the administration of three puffs of inhaled bronchodilator (salbutamol) from a metered dose inhaler (100µg/puff). Subjects used for this study were classified as asthma (self-reported doctor-diagnosed asthma), normal (no history of airway disease, normal lung function) and COPD (FEV₁/FVC <0.7 and FEV₁ <80% predicted).

The in vivo response to DI was calculated as a ratio of the expiratory flow at 60% of the vital capacity (0.6 FVC) measured following maximal (M) inspiration, to that measured after a sub-maximal or partial (P) inspiration, the M/P ratio (Section 2.3.2). An M/P
ratio of >1 indicates that flow is increased following a DI, suggesting bronchodilatation, while an M/P ratio <1 indicates that flow was decreased following a DI, suggesting bronchoconstriction. (Figure 8.1)

Figure 8.1: Flow-volume traces from subjects with M/P ratios less than 1 (A), greater than 1 (B) and equal to 1 (C). Volumes are relative not absolute.
8.2.2 Structural Assessment
Post-operative tissue was collected and fixed for estimation of the area of ASM ($A_{ASM}$), numerical density of ASM cells ($N_V$), mean ASM cell volume ($V_C$), the number of ASM cells per unit length of airway ($N_L$) (30µm, haematoxylin), and estimation of the volume fractions ($V_V$) of ASM, ECM and Other within the ASM layer (0.5µm, Masson’s Trichrome technique).

8.2.3 Statistics
ANOVA was used to compare the M/P ratios in subjects with asthma and COPD with subjects who had neither asthma nor COPD. Correlation analysis (Pearson’s correlation) was performed to determine the relationship between M/P ratios and subject demographics and parameters of the ASM layer.

8.4 Results

8.4.1 Subject Characteristics
There were more females than males in the asthmatic group and more males than females in the COPD groups (Table 8.1). Subjects without COPD tended to be younger although this was not statistically different ($p = 0.105$). Cigarette smoking (current smokers, never smokers or ex-smokers) was similar between groups. There was a significant difference between the post-bronchodilator FEV$_1$/FVC ratio of normal and COPD subjects ($p = 0.01$) but not for FEV$_1$% predicted between groups ($p = 0.55$). The M/P ratios for subject groups are presented in Table 8.2. These were typically <1 in the asthmatic and COPD groups indicating bronchoconstriction following a DI. Normal subjects typically had an M/P ratio close to one, indicating no effect of DI. In subjects with COPD, the M/P ratios were less compared with the normal group before and after bronchodilator (Table 8.2). A similar trend was also observed in the asthma group, but this was not statistically different.
Table 8.1: Subject characteristics and lung function.

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Gender</th>
<th>Age</th>
<th>Smoking</th>
<th>FEV₁ % Pred</th>
<th>FVC % Pred</th>
<th>FEV₁/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4 / 3</td>
<td>54 ± 14</td>
<td>2 / 3 / 2</td>
<td>76.2 ± 4.9</td>
<td>75.8 ± 7.6</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>Asthma</td>
<td>1 / 6</td>
<td>63 ± 11</td>
<td>1 / 2 / 3</td>
<td>83.6 ± 13.2</td>
<td>95.6 ± 8.6</td>
<td>0.68 ± 0.11</td>
</tr>
<tr>
<td>COPD</td>
<td>8 / 2</td>
<td>71 ± 4</td>
<td>4 / 1 / 5</td>
<td>77.0 ± 18.0</td>
<td>86.6 ± 19.6</td>
<td>0.65 ± 0.04</td>
</tr>
</tbody>
</table>

Table 8.2: M/P ratios of all subject groups indicating

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>M/P Ratio Pre-bronchodilator</th>
<th>M/P Ratio Post-bronchodilator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.03 ± 0.31</td>
<td>1.00 ± 0.34</td>
</tr>
<tr>
<td>Asthma</td>
<td>0.75 ± 0.21</td>
<td>0.73 ± 0.14</td>
</tr>
<tr>
<td>COPD</td>
<td>0.58 ± 0.20*</td>
<td>0.59 ± 0.33*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. normal

8.4.2 Relationship of M/P Ratio to Lung Function

When all subjects were combined, the pre-bronchodilator M/P ratio was inversely related to age (Figure 8.2A) but not to subjects height (p = 0.945, r = -0.017) or weight (p = 0.416, r = 0.193), and positively correlated with the FEV₁/FVC ratio (Figure 8.2B). Pre-bronchodilator M/P ratio was positively correlated with Nᵥ (Figure 8.3) but not with Vᵥ (p = 0.275, r = -0.250) or the VᵥASM (p = 0.326, r = 0.225), VᵥECM (p = 0.058, r = -0.320) or Vᵥother (p = 0.869 r = -0.038). There was a trend towards a positive correlation between pre-bronchodilator M/P ratio and N₅, but this did not reach statistical significance (p = 0.094). There was no relationship with post-bronchodilator M/P ratios for subject age (p = 0.701, r = -0.102), height (p = 0.387, r = -0.232) or weight (p = 0.901, r = -0.034). Post-bronchodilator M/P ratio was not related to FEV₁% predicted (p = 0.102, r = 0.423), FVC% predicted (p = 0.399, r = 0.266), FEV₁/FVC (p = 0.086, r = 0.422) or any measured ASM parameter (Table 8.3).
Figure 8.2: Pre-bronchodilator M/P ratio related to subject age (A) and lung function (FEV$_1$/FVC; B).

Figure 8.3: Pre-bronchodilator M/P ratio related to the number of ASM cells per volume ($N_V$).
Table 8.3: Correlation coefficients for post-M/P ratio and ASM parameters.

<table>
<thead>
<tr>
<th>ASM parameter</th>
<th>p-value</th>
<th>r-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_C (μm³)</td>
<td>0.249</td>
<td>-0.306</td>
</tr>
<tr>
<td>N_L (cells/mm)</td>
<td>0.557</td>
<td>0.159</td>
</tr>
<tr>
<td>N_V (cells/mm³)</td>
<td>0.159</td>
<td>0.369</td>
</tr>
<tr>
<td>V_{VASM}</td>
<td>0.968</td>
<td>-0.012</td>
</tr>
<tr>
<td>V_{VECM}</td>
<td>0.571</td>
<td>-0.153</td>
</tr>
<tr>
<td>V_{VOther}</td>
<td>0.717</td>
<td>0.099</td>
</tr>
</tbody>
</table>

Abbreviations; N_V = numerical density; V_C = mean volume of ASM cells; N_L = number of ASM cells per unit length of airway; V_{VASM} = volume fraction of ASM; V_{VECM} = volume fraction of ECM; V_{VOther} = volume fraction of “other” within the ASM layer.

8.5 Discussion

This chapter examined the in vivo response to DI (M/P ratio) in subjects with asthma and COPD and examined the relationship with the structure of the ASM layer. As expected, M/P ratio was less in subjects with asthma and those with obstructive disease (although this was not statistically significant in the asthma group). We found that most subjects with obstructive disease had an M/P ratio of <1 and that the absolute ratio was inversely related to subjects’ age. The pre-bronchodilator (prior to inhaled salbutamol) M/P ratio was positively correlated with the number of ASM cells per volume but not with any other ASM parameters.

DI is commonly thought to produce a bronchodilator response in healthy subjects and this is reduced in disease (Brusasco, Crimi et al. 1999; Crimi, Pellegrino et al. 2002; Jensen, Atileh et al. 2001) and may in fact cause bronchoconstriction (Pellegrino, Violante et al. 1991; Spicuzza, Ciancio et al. 2003), especially in subjects with poorly controlled asthma (Gibbons, Sharma et al. 1996; Pellegrino, Violante et al. 1996). In the present study, the majority of subjects studied (20/24 subjects pre-bronchodilator and 21/24 post-bronchodilator) had M/P ratios of less than one suggesting that the airway constricted after DI. In subjects with normal lung function, the M/P ratio was approximately 1 showing no evidence of bronchodilation in this group. There are several factors that need to be considered which may explain the lack of a
bronchodilator response to DI. Subjects studied are not healthy individuals per se since many had a history of smoking and all were undergoing surgery to remove pulmonary neoplasms. A history of smoking will likely reduce the bronchodilator effect of DI (Scichilone, Marchese et al. 2004; Scichilone, Bruno et al. 2005). It is unlikely that the small, peripheral lung tumours present in most of our subjects will have affected the response to DI. Perhaps more important is the lack of existing ASM tone in our subjects prior to the DI manoeuvre. Most studies assessing M/P ratio did so in patients after inhalation of a constricting agent (such as methacholine) (Burns, Taylor et al. 1985; Brusasco, Crimi et al. 1999; Crimi, Pellegrino et al. 2002; Jensen, Atileh et al. 2001; Scichilone, Marchese et al. 2004). In the presence of ASM tone airway hysteresis is greater and the M/P ratio increases further above one. In our study subjects did not receive a bronchial challenge as it was important to get a reliable post-bronchodilator FEV₁ to determine the presence of fixed airflow obstruction. Consequently, all M/P ratios were assessed in subjects with minimal airway tone (endogenous tone only), which would favour low values.

The majority of subjects (even some control subjects) tended to constrict after DI (M/P ratio <1) which is unexpected but can be easily explained. Similar to the airway, the lung parenchyma also exhibits hysteresis. The consequence of lung hysteresis is that after deflation, transpulmonary pressure at a given volume is less. If the transpulmonary pressure is reduced after DI, then the distending pressure on the airway wall is less, favouring airway constriction. This discussion is in line with the relative airway-parenchymal hysteresis theory presented by Hughes (Hughes, Hoppin et al. 1970; Burns, Taylor et al. 1985; Ingram 1987). Thus, in subjects with minimal airway tone, parenchymal hysteresis dominates airway hysteresis and favours bronchoconstriction, resulting in an M/P ratio of <1.

The M/P ratio was also inversely correlated with age. This has previously been observed by Scichilone (Scichilone, Marchese et al. 2004) in healthy subjects who performed DI following a challenge with inhaled methacholine. In the scenario where parenchymal hysteresis dominates airway hysteresis (present study), age-related changes in parenchymal mechanics may explain the fall in the M/P ratio. Changes in lung mechanics with age are well documented, reflected by a decrease in FEV₁ (Fletcher and Peto 1977; Sherrill, Guerra et al. 2003; James, Palmer et al. 2005), lung
elastic recoil (Verbeken, Cauberghs et al. 1992) and changes in connective tissue structures (Verbeken, Cauberghs et al. 1992). As discussed, parenchymal hysteresis reduces the distending pressure on the airway wall. In the aged lung, a loss of lung elastic recoil may reduce airway distending pressure further and result in a lower M/P ratio. Our subjects with COPD tended to be older which may have contributed to a lower M/P ratio (Scichilone, Marchese et al. 2004).

An original finding of this study is that the M/P ratio was related to the ASM volume density, and there was a similar trend with the number of ASM cells per length of airway (p=0.094). Since the M/P ratio is a balance between airway and parenchymal hysteresis, it is possible that airway hysteresis was greater in airways that contain a greater density of ASM cells in the ASM layer. Airways that contain a greater density of ASM cells may express greater ASM tone, favouring airway hysteresis and a higher M/P ratio, although not to the extent that it exceeds one. In support of this, there was no relationship between ASM cell volume density and cells per length of airway with M/P ratio after inhaled bronchodilator where ASM tone should be at least partially reversed.

Other than the relationships discussed above there were no associations between ASM structure and M/P ratio. This suggests that non-muscle airway wall structures play a greater role in determining the response to DI in the absence of induced ASM tone. Alternatively, under conditions where parenchymal hysteresis may dominate (as is suggested to occur in our subjects) ASM structure may not play a major role. Follow-up studies should examine a possible relationship between M/P ratio and parenchymal structure, which was beyond the scope of this thesis.

In concluding, while experiments on isolated airways segments in vitro provide many insights into airway physiology (Chapters 5 and 7), interactions with the surrounding parenchyma should be taken into account when interpreting their significance to the in vivo scenario. In the absence of ASM tone, the airway response to DI may be less dependent on the ASM layer but on the properties of the parenchyma which may change in disease or in the aged lung. Future studies should determine whether there is an association between the response to DI and structural characteristics of the ASM layer in the presence of induced ASM tone.
9.1 Aims and Outcomes

This thesis has examined the structure and function of the airways in subjects with asthma and fixed airflow obstruction – chronic obstructive pulmonary disease (COPD), focusing on the role of the smooth muscle layer in disease pathogenesis. The thickness (area or volume) of the ASM layer is known to be increased in both asthma (Heard and Hossain 1973; Ebina, Yaegashi et al. 1990; James 1997; Woodruff, Dolganov et al. 2004; Regamey, Ochs et al. 2008) and COPD (Hogg, Chu et al. 2004; Kuwano, Bosken et al. 1993; Saetta, Di Stefano et al. 1998) and may be the most important factor determining the degree of airway narrowing in these airway diseases (Wiggs, Bosken et al. 1992; Postma and Kerstjens 1998; Oliver, Fabry et al. 2007). Hypertrophy or hyperplasia of ASM cells, an increase in the volume fraction of ECM or a combination of these processes may all contribute to the increase in the thickness of the ASM layer and can be quantified using stereological techniques. The mechanisms for the increase in the thickness of the ASM layer may differ in asthma compared with COPD and contribute to known differences in the functional behaviour of the airway in patients with asthma and COPD. We have studied the ASM layer in asthma and COPD to determine the relative contributions of ASM cell size and number, and ECM to the composition of the ASM layer. The primary aims of this thesis were to:

1. Refine methods for the stereological measurement of the composition of the ASM layer in situ by examining the sources of variation that exist within and between airway segments.
2. Assess ASM cell hypertrophy and hyperplasia and ECM volume fraction within the ASM layer in normal subjects and in patients with asthma and COPD.
3. Relate ASM and ECM parameters and dimensions of the airway wall (areas of smooth muscle, cartilage, total wall, mucous glands and epithelium; basement membrane thickness) and subject characteristics (age, sex, duration and severity of asthma, cigarette smoking and allergies) to the diagnosis of asthma.
4. Relate ASM and ECM parameters and subject characteristics (age, sex, cigarette smoking) to the diagnosis and severity of COPD.
5. Relate in vitro physiological properties of the isolated airway wall (airway narrowing and bronchodilatory response to DI) to ASM and ECM parameters and airway dimensions.

6. Relate the bronchodilator response to DI in vivo to ASM and ECM parameters and airway dimensions.

9.2 The Burden of Chronic Airway Disease and Limitations in Therapy

Chronic airway disease is a significant source of mortality and morbidity, with 3,250,000 deaths worldwide in 2012 attributed to asthma and COPD (WHO 2004; GINA 2012; WHO 2012). In Australia alone approximately 20% of adults report having ever been diagnosed with asthma with over 10% displaying current symptoms (ACAM 2008). COPD is responsible for approximately 8% of respiratory deaths and 4% of all deaths in Australia every year (ACAM 2011; AIHW 2012). Smoking is commonly associated with the development of COPD and the with reduced lung function, airway disease and emphysema are often not diagnosed until later in life. Thus, the prevalence of COPD worldwide is expected to increase as the population ages and if current smoking rates persist. The morbidity and mortality associated with asthma and COPD puts substantial burden on national health care systems (Rabe, Hurd et al. 2007).

Current therapies for the treatment and prevention of asthma and COPD have reached a plateau in reducing the burden of disease in developed countries. In asthma, pharmacological intervention with bronchodilator and anti-inflammatory agents are generally effective in controlling symptoms (Hoshino, Toda et al. 2009; Bateman, Hurd et al. 2008) but for the most part do not treat the underlying structural abnormalities. While there is evidence that inhaled corticosteroids reduce reticular basement membrane thickness (Hoshino, Nakamura et al. 1998; Lapperre, Sont et al 2007) it is unknown if this treatment can affect the increased thickness of ASM layer. Longitudinal studies suggest that current treatments do not alter the development of fixed airflow obstruction or the rate of decline in lung function (CAMP 2000).

Similar to asthma, treatment for COPD addresses symptoms rather than the underlying cause. There are currently no treatments for COPD that consistently improve FEV$_1$ toward normality, with smoking cessation the only intervention that reliably reduces the
rate of decline in lung function (Anthonisen, Connett et al. 1994) and mortality rate (Anthonisen, Skeans et al. 2005). Bronchodilator and corticosteroid or long-acting anti-cholinergic agents show only modest improvements in lung function (FEV₁), quality of life and exacerbation rates (Calverley, Pauwels et al. 2003; Tashkin, Celli et al. 2008). The relative ineffectiveness of such approaches in COPD (defined as fixed airflow obstruction which is non-responsive to bronchodilators and corticosteroids) may not be surprising as these pharmacological agents are essentially ‘borrowed’ from therapies used in asthma, making the underlying but likely incorrect assumption that mechanisms for these diseases are related.

Distinct functional differences exist in airway structure and mechanics between asthma and COPD. Asthma is generally classified as “reversible airflow obstruction”, responding well to bronchodilators. In comparison, COPD is associated with “irreversible” airway obstruction, responding poorly to bronchodilators and strongly associated with cigarette smoking. While airway hyperresponsiveness is identified in both asthma and COPD, the extent of airway narrowing to a contractile stimulus is considerably greater in asthma.

Before commenting further on the major findings on airway pathology in asthma and COPD arising from this thesis, I will briefly discuss the use of stereology for the study of airway disease and highlight considerations for the different staining techniques used in this thesis.

9.3 Stereology for the Study of Airway Disease

Stereology is the most reliable, accurate and informative technique to study the structure of the ASM layer in health and disease. Stereology allows for quantitative estimation of an object of interest within a known volume without relying on assumptions of the shape, size, orientation or distribution of the object of interest. Stereology was employed throughout this thesis, specifically using the ‘optical dissector’ to estimate ASM cell size and number. This technique is an extension of the ‘physical dissector’ that has been used in previous studies to assess the relative contributions of ASM hypertrophy and hyperplasia (Heard and Hossain 1973; Ebina, Yaegashi et al. 1990; Ebina, Takahashi et al. 1993).
The optical and physical dissectors can both be used to estimate the volume density of ASM cells by counting the number of ASM nuclei (ASM cells are mononuclear) within the smooth muscle layer to assess ASM hypertrophy or hyperplasia. The physical dissector has been used in studies of human airways (Heard and Hossain 1973; Ebina Takahashi et al. 1993) but is labour intensive, requiring that all sampled ASM nuclei be counted using pairs of parallel planes (sequential tissue sections) separated by a known distance. The uppermost plane is the 'reference' section and the lower is the 'look-up' section. Rules require only those particles that appear on the reference section, but not the look up section to be counted (Gundersen 1977; Mayhew and Gundersen 1996). Each dissector pair is randomly located within the specimen of interest such that the counting process needs to be repeated multiple times within the specimen for a particle count to be obtained. The major advantage of the optical dissector over the physical dissector is that the same outcomes can be achieved in a comparatively shorter time, although only in translucent material such as tissue sections. The optical dissector uses thick sections of airway and the investigator need only to scan through successive focal planes without the need for serial sectioning. ASM nuclei can then be counted within this known volume of tissue while employing specific counting rules (Gundersen 1977; Gundersen, Bagger et al. 1988). If employed correctly, the number of ASM can be quickly and accurately estimated making larger studies and greater sample sizes more feasible. By use of the optical dissector, this thesis successfully studied over 60 ‘healthy’ and 160 ‘diseased’ subjects to detect ASM hypertrophy and hyperplasia in asthma and COPD.

Recognising that in the majority of human studies, only a limited number of samples are available, the first aim of this thesis was to assess sources of variability within and between samples. We have determined (using a random sampling technique within airway segments) the extent to which a single section taken from a length of airway is a representative sample, in large and small airways. We found that airway size was the most significant source of variation, necessitating the estimation of ASM parameters on both large and small airways segments. The number of sampling regions and the thickness of tissue sections also affected estimations of ASM cell numerical density, volume fractions of ASM and ECM and estimation of the area of the ASM layer. The findings from these experiments formed the basis for recommendations on sampling frequency and the methods used in subsequent chapters of this thesis.
In summary, when using the optical disector to study the ASM layer, I make the following recommendations:

1. Appropriate matching of airway size between groups – both “large” and “small” airways, with data to be analysed within specified airway sizes.
2. For estimates of ASM nuclei volume density, use >40 high-power sampling fields within the same airway section.
3. For estimates of tissue volume fractions within the ASM layer, use sections of <1µm thickness.
4. For estimations of area of ASM, sections between 4µm or 30µm thickness give equivalent results.

9.4 Limitations of Staining Techniques.

This thesis has used a number of different stains and staining techniques to quantify ASM cell size, number and the contribution of the ECM to the ASM layer. The main stains employed were haematoxylin, haematoxylin and eosin and the Masson’s Trichrome technique. While for the most part these stains were appropriate to address the specific aims of the thesis, there are limitations that require consideration.

9.4.1 Identifying Airway Smooth Muscle Nuclei

Haematoxylin staining was used for the identification and counting of ASM nuclei within the smooth muscle layer. However in haematoxylin-stained tissues imaged by light microscopy, it is exceedingly difficult to distinguish nuclei of smooth muscle from that of myofibroblasts. There are currently no stains or immuno-histochemical markers that clearly distinguish myofibroblasts and myocytes. Myofibroblasts do not express specific markers (Begueret, Berger et al. 2007) and stain positive for both smooth muscle actin and vimentin antigens. Electron microscopy may be used to identify myofibroblasts using ultrastructural characteristics such as rough endoplasmic reticulum and poorly developed myofilaments (Eyden 2001). Myofibroblasts are present within the ASM layer in both asthmatic and non-asthmatic subjects (Begueret, Berger et al. 2007), however their ability to effectively differentiate into actual ASM and contribute to hyperplasia in asthmatic subjects requires further investigation. The present thesis, like many studies before, makes the assumption that the degree of differentiation of
contractile cells within the ASM layer is the same, although this is yet to be determined. Similarly, the phenotype (contractile, proliferative or secretory) of the smooth muscle cells within the ASM layer remains the subject of current debate and investigation. In examining the ASM layer, we find the vast majority (>90%) of the nuclei are elongated and are readily differentiated from endothelial cell nuclei, from mast cell nuclei (the next most common cell type in the ASM layer) and from the occasional lymphocyte or granulocyte.

9.4.2 Quantifying the Extracellular Matrix
The Masson’s Trichrome technique has been used throughout this thesis to estimate the volume fraction of ASM and ECM within the ASM layer. The technique of quantifying ECM on very thin sections (Section 9.3) has been developed in our laboratory for minimising overlap of tissue components. The use of transverse tissue sections also conserves airway structure and allows contiguous thin and thick airway sections to be cut for additional morphology studies. The use of these techniques in this thesis have resulted in several publications (Page 3) however the ability of the Masson’s technique to stain all of the constituent proteins in the ECM is yet to be fully investigated.

Expert advice and basic histology textbooks refer to Masson’s techniques as staining “connective tissue”. According to Masson P. (Sheridan 1929) this technique separately identifies: nuclei, elastic fibres (blue-black); cytoplasm, muscle, acidophilic granules (red) and; collagen, cartilage, mucin, basophilic granules (blue-green). To the best of my knowledge, there are no studies to show whether this ‘collagen’ staining includes all ECM proteins types. As proper quantification of the ECM would require all ECM protein types to be stained, the suitability of Masson’s Trichrome in accomplishing this task awaits future investigation. Such investigations require quantification of the volume fractions of individual ECM proteins on sequential thin airway sections. The volume fractions of each ECM component (e.g. fibronectin, versican, biglycan, decorin and collagen) would then need to be totalled and compared with an airway section stained with the Masson’s Trichrome technique. Other ECM stains such as toluidine blue should also be tested in this manner to determine whether the ECM components can be successfully quantified without the use of more labour-intensive immunohistochemical staining techniques. This work is beyond the scope of this thesis.
9.5 Structural and Functional Differences between Asthma and COPD

9.5.1 Structural Changes in the ASM Layer

A major aim of this thesis was to determine the structure of the ASM layer in both asthma and COPD and whether differences exist between these diseases. While the smooth muscle layer appears to be superficially composed entirely of ASM cells, only approximately 75% is in fact muscle (Thomson, Bramley et al. 1996) with the rest comprised of ECM, space, inflammatory cells and blood vessels. Changes in the structure of the ASM layer may arise through a change in any of these compartments. A major finding of this thesis is that the pathological structural changes to the ASM layer are distinctly different in asthma and COPD. Table 9.1 summaries the respective structural pathologies of the ASM layer identified in this thesis for subjects with asthma or COPD.

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASM Hypertrophy</td>
<td>Large airways only</td>
<td>Neither large nor small airways</td>
</tr>
<tr>
<td>ASM Hyperplasia</td>
<td>Both large and small airways</td>
<td>Neither large nor small airways</td>
</tr>
<tr>
<td>Volume fractions of tissue components</td>
<td>Concomitant increase in absolute volumes of ASM and ECM in large or small airways without changes in volume fractions.</td>
<td>Increase ECM in large and small airways with no change in ASM</td>
</tr>
</tbody>
</table>

In the study of airways from subjects with asthma, tissue was obtained from a large cohort of subjects with severities of disease ranging from mild to very severe. These were predominantly adult subjects with the ages ranging from 14 – 67 years. There was a paucity of tissue from younger age groups which was a limitation of this thesis, particularly since 20% of all children aged 0 – 15 years are diagnosed with asthma (ACAM 2008). The early life structural origins of asthma are an important area of asthma research (Young, Le Souef et al. 1991; Turner, Young et al. 2009; Kusel, de Klerk et al. 2007; Sears, Greene et al. 2002; Robertson 2002) with quantification of ASM size and number thus far only performed in one study. Regamey (Regamey, Ochs et al. 2008) used bronchial biopsies from 24 asthmatic children (aged 7 - 16 years), and
showed ASM hyperplasia with a trend for increased cell size. Although a significant study, limitations exist with the use of biopsy specimens in regards to sample size, number of samples, and anatomical sampling location which may not be representative of a complete airway section or of smaller conducting airways (Carroll, Carroll et al. 2006; Gamble, Qiu et al. 2006; Jeffery, Holgate et al. 2003).

The main structural alterations in the layer of smooth muscle in the airways that was observed in asthmatic subjects were the presence of hyperplasia of ASM cells in the large, medium and small airways in fatal cases of asthma, and hypertrophy of ASM cells in the large airways in cases of both fatal and non-fatal asthma (Chapter 4). The first definitive study to demonstrate both ASM hypertrophy and hyperplasia in the airways from subjects with asthma was by Heard and Hossain in 1973 (Heard and Hossain 1973) albeit in a small subject group comprising only five mild to moderate asthmatic subjects. Heard and Hossain also adopted the stereological approach (the physical disector method) which had not been previously applied in studies of the ASM layer in asthma. This thesis extends these findings in a much larger cohort of patients (n=155).

Few studies on the ASM layer have been performed in COPD. Chapters 6 and 7 studied the composition of the ASM layer in both large and small airways from subjects with a range of severities of COPD. Findings suggest that in COPD the major structural abnormality of the smooth muscle layer is an increase in the volume fraction of the ECM rather than changes to the smooth muscle cells themselves, in contrast to what was observed in asthma. The positive relationship observed between pack-years of smoking and volume fraction of ECM suggests that exposure to cigarette smoke has a cumulative effect on remodelling of the ASM layer in COPD.

Changes in the ECM have been noted in other studies of COPD (Krimmer, Burgess et al. 2012; Kranenburg, Willems-Widyastuti et al. 2006; Annoni, Lancas et al. 2012) and may be related to disease severity (Black, Ching et al. 2008) however the volume fraction of ECM has not previously been examined. The observed increase in the volume fraction of ECM is supported by studies showing an increase in the staining intensity of specific ECM proteins in the airways of subjects with COPD (Kranenburg, Willems-Widyastuti et al. 2006; Annoni, Lancas et al. 2012). A decrease in ECM has
also been reported (Black, Ching et al. 2008; Annoni, Lancas et al. 2012). It is possible that the change in ECM protein type may be related to the severity of COPD. This could explain the increase in the fraction of ‘other’ in the ASM layer observed in mild COPD subjects in Chapter 6 which I propose is due to ECM that went undetected using the Masson’s Trichrome staining technique. In contrast to the findings of the thesis, one study has shown a mild degree of ASM hypertrophy in the large airways of COPD with the small airways remaining within the range of the controls (Ebina, Yaegashi et al. 1990). That study examined 13 subjects with mild COPD and may have overestimated the volume of ASM cells as ECM was not separately measured. It may have been the increased volume of ECM rather than muscle that contributed to the increased estimated average volume of airway smooth muscle cells. The increase in the volume fraction of the ECM that I have observed, if not corrected for, will lead to overestimation of ASM cell size.

9.5.2 Consequences for Airway Function
The different structural abnormalities observed in the ASM layer between asthma and COPD are significant for two reasons. Firstly, the pathological mechanisms producing the structural changes are likely to differ which is important when considering new therapies. Secondly, these structural differences (hypertrophy, hyperplasia or an increase in ECM) may contribute to the functional differences observed between asthma and COPD. The functional consequences of the remodelled ASM layer were examined by assessing the mechanical properties of isolated airway segments from subjects with asthma and from subjects without asthma with varied smoking histories and levels of lung function.

In asthma it is likely that increased thickness of the ASM layer (due to hyperplasia and hypertrophy of ASM cells) contributes to the excessive airway narrowing observed in both moderate and severe cases (Wiggs, Moreno et al. 1991; Moreno, Hogg et al. 1986). This thesis shows that maximal airway narrowing in isolated bronchial segment positively correlated with the area of the ASM layer in subjects with a history of doctor-diagnosed asthma. Airways from subjects with asthma had an increased area of the ASM layer which was associated with an increase in maximal airway narrowing. Mathematical simulations predict that increased total contractile proteins, which may occur due to either hypertrophy or hyperplasia, would result in more force development.
and thereby facilitate an increase in airway narrowing (Oliver, Fabry et al. 2007). The data presented in Chapter 5 presents the first biological evidence that increased ASM mass produces increased maximal airway narrowing in whole airway segments. These findings suggest that the airway hyperresponsiveness present in subjects with asthma partly involves an abnormality specific to the airway wall, i.e. the amount of ASM.

The present findings suggest that the increased thickness of the ASM layer previously demonstrated in subjects with COPD is due to an increase in the volume fraction of ECM within the smooth muscle layer with no change in the size or number in ASM cells. An inverse relationship was observed between the volume fraction of ECM and FEV₁% predicted (Chapter 6). For in vitro investigations, which were limited to subjects undergoing lung surgery, there was only a narrow range of lung function which was not appropriate for COPD classification using the GOLD criteria. Instead, we examined the functional consequences of airway remodelling using subjects’ smoking histories, since cigarette smoke exposure is the most common predictor of the development of COPD (Buist, McBurnie et al. 2007). In large bronchial segments, increased pack-years of cigarette smoking were associated with an increased volume fraction of ECM within the layer of smooth muscle in the airway with a trend for a decreased volume fraction of ASM. Smoking and volume fractions within the ASM also influenced the mechanical properties of the airway wall. Airway closing pressure (a measure of collapsibility) was inversely related to the volume fraction of ECM such that airways with a greater volume fraction of ECM required a more negative pressure to bring them to collapse. Similarly, reduced fractions of ASM lead to a reduction in airway wall compliance and a trend towards a more negative pressure to collapse. Therefore the changes to the composition of the ASM layer with smoking may stiffen airways and restrict movement of the ASM layer. In vivo, smoking-related remodelling of the ASM layer may contribute to the poor response to bronchodilator in COPD and the lesser response to contractile stimuli compared with asthma (Toelle, Xuan et al. 2013).

The evidence from the present thesis suggests that the functional abnormalities in asthma and COPD include structural abnormalities of the airway smooth muscle layer in large airways and concomitant changes in airway mechanics. These structural and functional abnormalities manifest differently in asthma compared with COPD and are
summarised in Figure 9.1. It is recognised that changes to the structure and function of the airway wall apart from the smooth muscle layer also contribute to airway diseases (Okazawa, Pare et al. 1994; Bai and Knight 2005; James and Wenzel 2007) however, this thesis has focused on the specific role of the ASM layer.

Figure 9.1: Proposed role of the smooth muscle layer in the large airways in asthma and COPD.

9.6 Origins of Asthma and COPD
The functional and structural data collected during this thesis suggest distinctly different mechanisms for the development and severity of COPD and asthma. As discussed, functional differences between asthma and COPD can be explained in part by structural changes to the ASM layer. There are also differences in the origin and development of these diseases. Most cases of asthma have their origins early in life and show surprising stability with regard to decrements in lung function, treatment requirements and, as we have observed, thickness of the ASM layer, in relation to clinical severity. COPD is predominantly a disease of adulthood associated with an accelerated rate of decline in lung function related to cigarette smoking although early life developmental changes, exposures and disease (including asthma) may contribute to fixed decrements in lung function.
While it was not possible to study changes to the ASM layer in subjects less than ten years of age due to tissue availability, our findings still suggest that the ASM layer changes little throughout adulthood and that ASM structure is determined early in life. Several studies of airway structure in children report features of remodelling in asthma to be present at a young age (Saglani, Malmstrom et al. 2005; Regamey, Ochs et al. 2008; Pohunek, Warner et al. 2005; Bossley, Fleming et al. 2012; Berankova 2013). Evidence of airway remodelling has been observed in children as young as four years when the clinical manifestations of asthma symptoms are not yet present (Berankova 2013). These data are consistent with the present observations that the amount of remodelling in adults subjects with asthma was not related to age, age of onset of asthma or duration of disease. The structural data also correspond well with longitudinal data on lung function. Childhood studies of asthma show that subjects with a life-long history of asthma have reduced baseline lung function at age 18 which can be tracked from infancy into childhood and then into adulthood (Robertson 2002; Sears, Greene et al. 2003; James, Palmer et al. 2005).

The implication of these data is that changes to the ASM layer may be present very early in the clinical course of the disease, perhaps even prior to birth, raising the possibility that structure is an independent risk factor/predictor of asthma. There are a number of mechanical and inflammatory pathways that can modulate the thickness of the ASM layer. In utero, smooth muscle first appears in the airways at approximately five weeks gestation (Sparrow and Lamb 2003) with the development of the ASM controlled by mesenchymal cell tension and paracrine morphogenic factors (Badri, Zhou et al. 2008). Numerous stimuli in utero may adversely affect the development of the ASM layer in the intrauterine environment resulting in increased numbers or size of ASM prior to birth (Henderson and Warner 2012; Duijts 2012). Maternal influences such as cigarette smoking, hypertension, pre-eclampsia and anxiety levels have all been associated with persistent wheeze in children (Rusconi, Galassi et al. 2007; Duijts, Jaddoe et al. 2012; Guxens 2013). Postnatally, thickening of the ASM layer is mostly attributed to allergen exposure (Bentley, Deng et al. 2009). Environmental factors and genetic susceptibility may also play a role in airway remodelling and subsequent development of asthma (Sonnenschein-van der Voort, de Kluizenaar et al. 2012; Macintyre EA 2013; Hao, Bosse et al. 2012; Bossé 2013). Whether the ASM layer remodelling observed in adults (ASM hypertrophy and hyperplasia) can be accounted
for by early life exposure to allergens and airway remodelling in children is currently unclear.

COPD unlike asthma, is most often regarded as a disease of adulthood. Reduced lung function leading to the diagnosis of COPD may occur due to genetic susceptibility (Wilk, Shrine et al. 2012; Balantic, Rijavec et al. 2013) and particularly ongoing exposures to inhaled toxins (e.g. cigarette smoke) (Buist, McBurnie et al. 2007). Cigarette smoke exposure is an acknowledged and important risk factor for the development of COPD (Buist, McBurnie et al. 2007). Early life events may contribute to the diagnosis of COPD in adulthood. As in asthma, maternal and environmental interactions can modify foetal airway growth which may subsequently affect lung function and the rate of decline of FEV₁ (Narang 2012). There is evidence that respiratory infections in childhood (Barker, Godfrey et al. 1991; Shaheen, Barker et al. 1995) and the presence of other lung diseases such as asthma, cystic fibrosis and bronchiectasis (Postma, Brusselle et al. 2012) are associated with an increased risk of developing COPD in later life. How the structure of the airways and the level of airway remodelling interact with early life and environmental exposures in the development of COPD requires further consideration.

Longitudinal studies have begun to show the lifetime influences that determine the level of FEV₁ at any stage of adult life (Robertson 2002; Sears, Greene et al. 2003; Mortimer, Neugebauer et al. 2008). Whether structural changes to the airways early in life determine the subsequent severity of COPD that will develop or whether there is a progression of “COPD” severity from mild to severe is currently unclear. This thesis highlights a role for cigarette smoking and its relation to the volume fraction of ECM and lung function. Since pack-years is a marker of duration of exposure, a positive correlation between ECM volume fraction and pack-years suggests that the structural and functional changes to the ASM layer are a progressive process with continued smoke exposure. It is possible that early life changes to the lung impact the initial development of COPD and the level of severity (Decramer and Cooper 2010) while subsequent biological and environmental factors impact the rate of decline of FEV₁ and subsequent disease progression (Narang and Bush 2012). Future studies are required to determine how smoking impacts the amount and type of ECM within the ASM layer.
9.7 Final Remarks
This thesis has demonstrated that the remodelling of the ASM layer is due to different mechanisms in asthma and COPD and that these differences in remodelling result in differences in airway function. We have established that although they may have some functional and structural similarities, asthma and COPD are distinctly different airway diseases with unique changes to the ASM layer. With knowledge of the mechanisms by which ASM layer remodelling occurs potential new targets may be revealed for the treatment and management of these airway diseases.
REFERENCES


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McDonough J. E., Yuan R., Suzuki M., Seyednejad N., Elliott W. M., Sanchez P. G., Wright A. C., Gefter W. B., Litzky L., Coxson H. O., Pare P. D., Sin D. D.,


Turner S. W., Young S., Goldblatt J., Landau L. I. and Le Souef P. N. (2009). "Childhood asthma and increased airway responsiveness: a relationship that begins


APPENDIX 1: Conference Participation

Oral Presentations


Posters


5. Elliot J, **Jones R**, Mauad T, Abramson M, K McKay K, Bai T, Green F, James A. Distribution of increased airway smooth muscle thickness and airway inflammation...


12. James A, **Jones R**, Mauad T, Dolhnikoff M, Abramson M, McKay K, Bai T, Green F, Elliot J. Increased airway smooth muscle cell size and number are not related to age or age at onset of asthma. Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Perth, April 2011


16. Jones R, Elliot J, Carroll M, James A. Variation of tissue volume fraction in the smooth muscle layer along the length of airways. International; Colloquium on Lung and Airway Fibrosis, Busselton, November 2010


### APPENDIX 2: Glossary and Abbreviations

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatal Asthma</td>
<td>Subjects who died as a direct result of asthma.</td>
</tr>
<tr>
<td>Non Fatal Asthma</td>
<td>Non-respiratory cause of death, but with a history of asthma (doctor diagnosed or reported by next of kin).</td>
</tr>
<tr>
<td>Control</td>
<td>Normal lung function and no history of respiratory disease.</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease. Defined in this thesis using spirometry with a post-bronchodilator FEV₁/FVC ratio of &lt;0.7 and an FEV₁% predicted &lt;80%.</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>Larger ASM cells per unit length of airway.</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>Increased numbers of ASM cells per unit length of airway.</td>
</tr>
<tr>
<td>NV</td>
<td>Numerical volume density of airway smooth muscle cells.</td>
</tr>
<tr>
<td>VC</td>
<td>Airway smooth muscle cell volume.</td>
</tr>
<tr>
<td>NL</td>
<td>The average number of airway smooth muscle cells per unit length of airway.</td>
</tr>
<tr>
<td>VVASM</td>
<td>Volume fraction of airway smooth muscle within the airway smooth muscle layer.</td>
</tr>
<tr>
<td>VVECM</td>
<td>Volume fraction of extracellular matrix within the airway smooth muscle layer.</td>
</tr>
<tr>
<td>VVother</td>
<td>Volume fraction of other elements, including space between cells, blood vessels and inflammatory cells within the airway smooth muscle layer</td>
</tr>
<tr>
<td>AASM</td>
<td>Area of the smooth muscle layer</td>
</tr>
<tr>
<td>Pbm:</td>
<td>Perimeter of the basement membrane. Used to determine airway size.</td>
</tr>
<tr>
<td>Morphometry</td>
<td>In this thesis, morphometry refers to the quantitative measurement of the structure of the airways by delineation.</td>
</tr>
<tr>
<td>Stereology</td>
<td>Stereology is a method that uses random, systematic sampling to provide unbiased and quantitative data. It allows quantitative measures of a three-dimensional shape to be made on two-dimensional sections. E.g. Quantifying the numbers of smooth muscle nuclei within the airway smooth muscle layer on airway sections.</td>
</tr>
<tr>
<td>Compliance</td>
<td>Inverse of wall stiffness. Defined as the volume change per unit pressure.</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Collapsibility</td>
<td>The amount of pressure required to bring an airway to total closure and is a measure of the compressibility of the airway wall.</td>
</tr>
<tr>
<td>Distensibility</td>
<td>The capacity of the airway to be stretched (dilate) during inflation.</td>
</tr>
<tr>
<td>DI</td>
<td>Deep Inspiration. <em>In vivo</em> when a subject breathes to total lung capacity, or <em>in vitro</em> simulated by positive hydrostatic inflationary pressures.</td>
</tr>
<tr>
<td>Static</td>
<td>No tidal oscillation. Transmural pressure held fixed at 5 cmH₂O.</td>
</tr>
<tr>
<td>Dynamic</td>
<td>Simulating tidal oscillations at physiologically relevant lung volumes. Transmural pressure varied from 5 to 10 cmH₂O.</td>
</tr>
</tbody>
</table>
APPENDIX 3: Respiratory Symptom Questionnaire

SUBJECT DETAILS

DOB    19    URN

Sex  M  F

1  Have you had a respiratory infection in the last 3 weeks?

2  Have you used your controller medication in the last 4 hours (12 hrs LAβA)?

3  Have you had a cigarette in the last hour?

SECTION I: RESPIRATORY SYMPTOMS

1  BREATHLESSNESS

Do you get short of breath when hurrying on level ground or walking up a slight hill?

Do you get short of breath walking with other people your own age on level ground?

Do you need to stop for breath when walking at your own pace on level ground?

2  COUGH

Do you usually cough first thing in the morning?

Do you usually cough during the day or at night?

If YES, do you cough on most days for as many as 3 months each year?

3  PHLEGM

Do you usually bring up phlegm from your chest each morning?

Do you usually bring up phlegm from your chest during the day or night?

If YES, do you bring up phlegm like this on most days for as many as 3 months each year?
4 **RHINITIS**

Do you sneeze or get an itchy, runny nose?   

*If YES, do you get this during any particular season?*

5 **WHEEZE**

Have you ever had wheezing or whistling in your chest?   

If YES, was this in the last 12 months?   

If YES, was this in the last month?   

6 **CHEST TIGHTNESS**

Have you ever felt tight in the chest?   

If YES, was this in the last 12 months?   

If YES, was this in the last month?   

7 **PAST RESPIRATORY ILLNESS**

Has your Doctor ever told you that you had any of the following?   

*Bronchitis*   

*Pneumonia*   

*Pleurisy*   

*Asthma/Bronchial Asthma*   

*Hay fever*   

*Allergic Rhinitis*   

*Sinusitis*   

*Other Chest Trouble, including chest or hear surgery?*   

*If YES, specify, including years*
SECTION II: OTHER ILLNESSES

Have you had any other illnesses. If YES, please state ____________________________

SECTION III: SMOKING HISTORY

1. Have you ever smoked regularly as many as 7 cigs./wk (1cigar/wk or 1oz tobacco/month) for as long as 1 year?  
   If NO please go to Question 6.

2. Do you smoke now?  
   If NO, how long is it since you gave up smoking (in years)?

3. How old were you when you first started smoking regularly (in years)?

4. Do (did) you smoke manufactured cigarettes?  
   If YES-
   How many do/did you smoke per day on weekdays?  
   How many do/did you smoke per day on weekends?

5. Do you smoke hand-rolled cigarettes?  
   If YES, how much tobacco do/did you usually smoke per week in this way?

6. Passive smoking – How many people in your household smoke?  
   If you work outside the home, are you exposed to tobacco smoke at work?

SECTION IV: ASTHMA SEVERITY (only if applicable, otherwise cont Section V)

1. In the last 12 months, have you lost days from work/studies/or normal activities due to asthma?  
   If YES, how many days?
2. Have you ever been hospitalised for breathing difficulties or asthma?

   If YES, how many times?

When was your last hospital admission?

3. Have you ever used oral corticosteroids (e.g. Prednisolone, “cortisone”)?

   If YES, ever?

   If YES, in the last 12 months?

SECTION V: MEDICATIONS

1. Are you currently taking any medication for asthma or to help your breathing, including inhalers, aerosols, nebulisers, pills, capsules or tablets? (not nasal symptoms). If YES, which medications?

<table>
<thead>
<tr>
<th>Name</th>
<th>Dose/# puffs</th>
<th># Times/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iii</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. How often do you use your reliever medication?

   Every day?

   Most days?

   Some days?

   Occasionally

   Rarely
3. What other medications do you currently take?

<table>
<thead>
<tr>
<th>Name</th>
<th>Dose</th>
<th># Times/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td></td>
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<td>vi</td>
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<tr>
<td>vii</td>
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<td></td>
</tr>
</tbody>
</table>
APPENDIX 4: Skin Prick Test

The skin prick test has been performed in accordance with the Australasian Society of Clinical Immunology and Allergy Guidelines (ASCIA 2006). The specific procedure used for this thesis is detailed below. Skin prick testing was performed as part of the recruitment process for post-operative tissue collection. Patient consent was obtained prior to commencement of skin prick testing.

Procedure:

Subject:

1. Subject should be seated in a comfortable position and the procedure explained to the patient.
2. Ensure patient has not taken any antihistamine before the commencement of the allergen test.
3. The left forearm (area to be tested) should be exposed with no risk of clothing brushing across the test area and wiping the test

Allergen placement:

1. Mark positions for skin pricks by a grid. Placement of allergens specified below. Prick tests should be at least 2cm apart to avoid overlapping reactions and false-positive results.

<table>
<thead>
<tr>
<th>Grass Mix #7</th>
<th>Mould Mix #10</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. Pteronyssinus (dust mite)</td>
<td>A. Tenuis (mould)</td>
</tr>
<tr>
<td>Cat Dander</td>
<td>Histamine (10mg/ml)</td>
</tr>
<tr>
<td>Dog Hair</td>
<td>Negative Control (Saline)</td>
</tr>
</tbody>
</table>

2. Apply allergens from the dropper bottle; all drops can be deposited before commencing pricking. The drop on the tip of the dropper can be touched on the skin to transfer the liquid but the actual tip of the dropper should not touch the skin.
3. Apply a single scratch to the surface of the skin using a sterile lancet. A separate lancet must be used for each allergen.
4. Carefully blot allergen from the skin after 1 minute, ensuring allergens do not mix.
5. Instruct the patient not to scratch the area to avoid affecting results.

Results:
1. The positive control (histamine) and negative control (saline) should be read at 10 min after the skin has been scratched.
2. The allergen reaction should be read at 15 minutes after the skin has been scratched.
3. The standard and accepted method for quantifying the skin prick reaction is to measure the mean diameter of the wheal, using a ruler marked in mm (Figure 1).
4. For a circular wheal, one measurement of the diameter (in mm) is performed.
5. If ovoid or irregular, the longest and shortest perpendicular axis are measured and the numbers are added and divided by 2 (mean diameter).

![Figure 1: Schematic for measurement of allergy reactions following a skin prick test](image-url)
APPENDIX 5: Staining Protocols

**Haematoxylin (30µm sections)**

Procedure:
1. Bring through three changes of xylene - 3 minutes in each solution.
2. Bring through three changes of 100% Ethanol – 1.5 minutes in each solution.
3. 90% Ethanol - 1.5 minutes
4. 70% Ethanol - 1.5 minutes
5. Rinse in distilled water.
7. Blue in running tap water for 5 minutes, rinse in distilled water.
8. 70% Ethanol - 1 minute
9. 90% Ethanol - 1 minute
10. Bring through three changes of 100% Ethanol – 1 minute in each solution.
11. Bring through three changes of xylene - 2 minutes in each solution.
12. Coverslip from Xylene.

NB: For de-waxing procedure (steps 1-4) on 0.5µm and 4µm sections, 2 minutes in each Xylene and 1 minute in each Ethanol solution.

**Haematoxylin and Eosin (4µm sections)**

Procedure:
1. Bring through three changes of xylene - 3 minutes in each solution.
2. Bring through three changes of 100% Ethanol – 1.5 minutes in each solution.
3. 90% Ethanol - 1 minutes
4. 70% Ethanol - 1 minutes
5. Rinse in distilled water.
6. Myers Haematoxylin - 4 minutes.
7. Blue in running tap water for 5 minutes, rinse in distilled water.
8. Eosin - 2 minutes
9. 70% Ethanol - 1 minutes
10. 90% Ethanol - 1 minutes
11. Bring through three changes of 100% Ethanol – 1 minute in each solution.
12. Bring through three changes of xylene - 2 minutes in each solution.
13. Coverslip from Xylene.

**Masson’s Trichrome (0.5µm sections)**

Procedure:
1. De-Wax tissue and sit in Bouin's solution overnight.
2. Wash in running tap water to remove the picric acid, 5 minutes.
3. Weigert's working hematoxylin, 10 minutes.
4. Blue in running tap water for 5 minutes, rinse in distilled water.
5. Biebrich scarlet for 5 minutes.
6. Rinse in distilled water.
7. Phosphotungstic/phosphomolybdic acid for 10 minutes - discard solution.
8. Transfer directly into Aniline blue for 5 minutes.
9. Rinse in distilled water.
10. 1% Acetic acid for 1 minute, discard solution, rinse in distilled water.
11. Dehydrate in air overnight, clear in xylene, and coverslip.