Structural and mechanical origins of fixed airflow limitation

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BSc (Honours) Medical Bioscience

This thesis is presented for the degree of Doctor of Philosophy of
The University of Western Australia
School of Human Sciences
2018
Acknowledgments

First, I would like to thank my supervisors: Dr Peter McFawn, Dr Peter Noble and Dr Alan James.

Dr Peter McFawn for his guidance, support, encouragement and moments of brilliance. Your long discussions on airway physiology, life and everything else, will be missed!

Dr Peter Noble, my mentor, for his advice, encouragement, guidance and unwavering belief in me. Words cannot express my gratitude for your support throughout these years!

Dr Alan James for his endless encouragement, guidance, advice and opportunities given to me.

I would also like to thank the following people, who, without their support and encouragement, this would not be possible.

Mr John Elliot for his advice, guidance and expertise on airway histology provided to me.

Dr Kimberley Wang for her expertise and help provided throughout the writing of this thesis. But more importantly, for your friendship.

James Wong, my fellow PhD student and friend. Thank you for your help and encouragement always.

Technical staff at CellCentral, Ms Mary Lee and Ms Shirley Chang, for their guidance and support.

Finally, I would like to thank the people closest to me:

Nicole Denteith, my friend, for her support during this time. Thank you for always being there to help with the kids when I could not be there. You know firsthand the sacrifices made to get to this point.

My parents, for providing me with the opportunities needed to become anything I set out to do.

My children, Ethan and Emily, for their infinite love and understanding. I hope I’ve set an example to show you that with hard work and dedication, you can accomplish anything in life.

Last but not least, my husband and best friend, Leigh. Thank you for your endless supply of love, support, encouragement and understanding. You were my sounding board for all my frustrations throughout this time.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship. It was also supported by an Ad-hoc scholarship funded by NHMRC (Project grant number APP1063068) and Dr Peter McFawn.
Thesis declaration

I, Alvenia Cairncross, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material which has been submitted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text and, where relevant, in the Declaration that follows.

The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

The research involving human data reported in this thesis was assessed and approved by the Department of Health, Western Australia, Human Research Ethics Committee (Approval number 2013-227) and recognised by The University of Western Australia Human Research Ethics Committee (Reference number RA\4\1\7200).

Written patient consent has been received and archived for the research involving patient data reported in this thesis.

The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee. Approval numbers APP513842; RA\3\100\945.
The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

The following approvals were obtained prior to commencing the relevant work described in this thesis. Approval numbers: 2007-161; 2013-227; 2015-53.

The work described in this thesis was funded by National Health and Medical Research Council Project grant numbers APP1063068 and APP513842.

This thesis contains published work which has been co-authored.

Signature:

Date: 22 June 2018
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### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1.3L&lt;sub&gt;ref&lt;/sub&gt;</td>
<td>Length stretched 1.3 times the reference length</td>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyper-responsiveness</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
</tr>
<tr>
<td>ASMR</td>
<td>Australian Society for Medical Research</td>
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<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DCLO</td>
<td>Diffusion capacity of the lung for carbon monoxide</td>
</tr>
<tr>
<td>DI</td>
<td>Deep inspiration</td>
</tr>
<tr>
<td>DRC</td>
<td>Dose-response curve</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal response</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in 1 s</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; %pred</td>
<td>Forced expiratory volume in 1 s as a percentage of the predicted value</td>
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<tr>
<td>FRC</td>
<td>Functional residual capacity</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative for Chronic Obstructive Lung Disease</td>
</tr>
<tr>
<td>His</td>
<td>Histamine</td>
</tr>
<tr>
<td>HRCT</td>
<td>High-resolution computed tomography</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LLL</td>
<td>Left lower lobectomy</td>
</tr>
<tr>
<td>LLN</td>
<td>Lower limit of normal</td>
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Lo  Optimum length for contraction
L_{ref}  Reference length
LUL  Left upper lobectomy
MANOVA  Multiple analysis of variance
M/P ratio  Maximal / Partial ratio
MCh  Methacholine
NHMRC  National Health and Medical Research Council
OCT  Optical coherence tomography
P_{bm}  Perimeter of the basement membrane
pD_{2}  negative logarithm of dose producing half maximal response
PD_{20}  Provocative dose producing a 20% fall in FEV_{1}
P_{L}  Elastic recoil pressure of the lung
P_{tm}  Transmural pressure
P_{tp}  Transpulmonary pressure
RLL  Right lower lobectomy
RML  Right middle lobectomy
RUL  Right upper lobectomy
RV  Residual volume
SEM  Standard error of the mean
TGF-ß  Transforming growth factor-beta
TLC  Total lung capacity
TSANZ  Thoracic Society of Australia and New Zealand
UWA  University of Western Australia
V_{0}  Volume of the fully relaxed airway at 0 cmH_{2}O
V_{5}  Volume of the airway at 5 cmH_{2}O
VC  Vital capacity
<table>
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<tr>
<th>Symbol</th>
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<tr>
<td>$V_{VASM}$</td>
<td>Volume fraction of airway smooth muscle</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>Volume fraction of extracellular matrix</td>
</tr>
<tr>
<td>$V_{VOTHER}$</td>
<td>Volume fraction of “Other”</td>
</tr>
<tr>
<td>$W_{ai}$</td>
<td>Inner wall area</td>
</tr>
<tr>
<td>$W_{ao}$</td>
<td>Outer wall area</td>
</tr>
<tr>
<td>$W_{at}$</td>
<td>Total wall area</td>
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List of publications

One chapter in this thesis was published in a peer-reviewed journal in the form of one research article listed below:

Chapter 5: Hyperinflation of bronchi in vitro impairs bronchodilation to simulated breathing and increases sensitivity to contractile activation.


I have also published previous experiments in peer-reviewed journals in the form of research articles listed below that has not arisen from this thesis:


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


L Qingyun, K Karnowski, PB Noble, A Cairncross, AL James, M Villiger, DD Sampson. Robust reconstruction of local optic axis orientation with fiber-based polarization-sensitive optical coherence tomography. Biomedical Optics Express. November 2018; 9(11): 5437-5455
### Authorship declaration

This thesis contains work that has been published.

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<td>I examined the effect of simulated lung hyperinflation in porcine airways. Results suggests that hyperinflation may directly impact airway function by reducing the effects of deep inspiration.</td>
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<tr>
<td>Chapter 5</td>
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<td>For this publication, I performed all the experimental work, including tissue collection, in vitro experimentation, manuscript preparation and final submission. Dr P.K McFawn assisted with study design, data analysis and manuscript preparation. Dr P.B Noble was instrumental in study design, data analyses, tissue collection, final manuscript preparation and submission.</td>
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<tr>
<td>Peter K. McFawn</td>
</tr>
<tr>
<td>Date: 22 June 2018</td>
</tr>
<tr>
<td>Peter B. Noble</td>
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<td>Date: 22 June 2018</td>
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</table>

I, Peter K. McFawn, certify that the student statements regarding their contribution to the work listed above is correct.

Coordinating supervisor signature:  
Date: 22 June 2018
List of abstracts

Data in this thesis has been presented in various local, national and international conferences. The presented abstracts are listed below:


A Cairncross, F Sanai, BE McParland, GG King, PK McFawn, JG Elliot, AL James, PB Noble. *Intrinsic airway smooth muscle tone in peripheral airways is determined by the volume fraction of muscle and extracellular matrix.* Thoracic Society of Australia and New Zealand, Annual Scientific Meeting, Adelaide, Australia, March 2018.


JG Elliot, S Drew, A Cairncross, PB Noble, AL James. Volume fraction of Collagen IV is increased within the ASM layer in COPD. Thoracic Society of Australia and New Zealand, Annual Scientific Meeting, Canberra, Australia, April 2017.


A Cairncross, RL Jones, JG Elliot, HW Mitchell, PK McFawn, AL James, PB Noble. The response to deep inspiration in COPD is not associated with changes in airway structure or function. Postgraduate Research Expo, University of Western Australia, Perth, Australia, July 2015.

A Cairncross, RL Jones, JG Elliot, HW Mitchell, PK McFawn, AL James, PB Noble. The response to deep inspiration in COPD is not associated with changes in airway structure or function. Thoracic Society of Australia and New Zealand, Annual Scientific Meeting, Perth, Australia, June 2015.


PB Noble, A Cairncross, RL Jones, JG Elliot, HW Mitchell, AL James, PK McFawn. *Exaggerated airway narrowing is related to increased airway smooth muscle mass in bronchial segments from subjects with a history of asthma.* European Respiratory Society, International Conference, Barcelona, Spain, September 2013.

Thesis summary

Background

Chronic obstructive pulmonary disease (COPD) is a common respiratory disease characterised by irreversible airflow limitation, due to a number of pathological events affecting both the airways (large and small) and lung parenchyma. From a mechanical perspective, lumen obstruction is a balance between contraction of the airway smooth muscle (ASM) layer and opposing distending forces which relax the ASM, including deep inspiration (DI). In subjects with COPD, there is evidence for both excessive contraction of the ASM (i.e., airway hyper-responsiveness, ‘AHR’) and a reduced bronchodilatory response to DI. The mechanisms producing these functional impairments are uncertain, although could involve changes that are intrinsic to the airway (remodelling) or extrinsic (e.g., lung hyperinflation).

It has recently been shown that among the structural changes that occur to the airways in patients with COPD, there is an increase in the volume fraction of extracellular matrix (ECM) within the ASM layer. That is, while the ASM layer comprises of ASM cells, ECM and other components (space, inflammatory cells and blood vessels), there is an imbalance of these structural components in the airways of COPD patients where ECM components expand. Compositional changes to the ASM layer in COPD are in contrast to subjects with asthma where there is a proportionate increase in the ASM layer thickness, with no change in the volume fraction of ECM. Changes in the composition of the ASM layer will theoretically impact airway narrowing capacity and the bronchodilatory responses to breathing manoeuvres such as DI that are initiated by dynamic stretch of the ASM layer.
Aims

The specific aims of this thesis were:

1. Compare airway narrowing capacity and bronchodilatory response to DI in large bronchial segments from subjects with and without COPD and assess how these properties are affected by the volume fraction of ECM within the ASM layer.
2. Determine the effect of the volume fraction of ECM within the ASM layer on contractile properties of small airways from human subjects.
3. Relate *in vivo* airway responsiveness to the composition of the ASM layer, including the volume fraction of ECM.
4. Establish how hyperinflation affects the airway response to deep inspiration.

Scope

Experiments comprise a series of *in vitro* analyses of airway function in organ bath chambers, using large and small airways obtained post-operatively from patients undergoing lung resection surgery. *In vivo* spirometry was performed, and in some subjects, bronchial provocation data obtained. Following functional assessment, airway wall structure, most notably composition of the ASM layer, was assessed using point counting techniques. Additional supporting experiments made use of animal tissue (pigs) to examine the effect of simulated lung hyperinflation.

Results and conclusions

**Aim 1:** Results show that large airways isolated from subjects with COPD exhibit an exaggerated bronchoconstrictor response to acetylcholine compared with airways from subjects without airflow limitation. There was also a concomitant increase in the volume fraction of ECM within the ASM layer in subjects with COPD. Findings support mechanical changes to the airway wall in subjects with COPD that is potentially mediated by compositional changes to the ASM layer, contributing to the onset of airflow limitation.
**Aim 2:** Structural composition of the ASM layer in terms of proportion of ECM and ASM was related to intrinsic ASM tone in small peripheral airways: lower volume fraction of ASM within the ASM layer and greater volume fraction of ECM favoured reduced ASM tone. Loss of intrinsic ASM tone due to increased volume fraction of ECM within the ASM layer of patients with COPD could favour collapse of peripheral airways due to reduced rigidity (stiffness) and contribute to the lack of response to inhaled bronchodilator in these patients.

**Aim 3:** *In vivo* responsiveness to methacholine was positively correlated with increased proportion of ASM within the ASM layer, however there was no association with proportion of ECM. In addition to determining baseline lung function, these data provided initial proof of concept that acute and induced bronchoconstriction is potentially impacted by the composition of the ASM layer.

**Aim 4:** Hyperinflation stiffened the airway wall and reduced the bronchodilatory capacity of simulated DI. There was also an increase in airway sensitivity to acetylcholine under conditions of hyperinflation. Findings suggest that hyperinflation may directly alter airway function by reducing the protective effects of DI and initiating contraction at low doses of contractile stimuli.
CHAPTER 1: Literature Review

1.1 Introduction

The prevalence of chronic respiratory disease has markedly increased worldwide and is a significant source of morbidity and mortality (Mannino, 2002, Mathers and Loncar, 2006). Chronic respiratory disease comprises a group of diverse conditions that includes pulmonary fibrosis, cystic fibrosis, asthma and chronic obstructive pulmonary disease (COPD). Pulmonary fibrosis is a restrictive interstitial chronic lung disease, often of idiopathic origin, and its commonest form, usual interstitial pneumonitis, has a median survival rate of only three-five years from the time of diagnosis (Ley et al., 2012). Pulmonary fibrosis is a relatively uncommon restrictive lung condition characterised by reduced lung compliance with reduced lung volumes and reduced gas exchange. It is associated with an increased rate of decline in lung function with mortality remaining high as there currently is no cure available (Raghu et al., 2015). Cystic fibrosis is caused by an inherited genetic mutation of chloride channels (Riordan et al., 1989), leading to the accumulation of thick viscous mucus in the lungs, airflow limitation, recurrent respiratory infections and reduced life expectancy. Another common respiratory disease is asthma, characterised by increased responsiveness of the airways to various stimuli. This is manifested as airflow limitation due to variable and excessive airway narrowing that can change spontaneously or in response to treatment over a short period of time (Wiggs et al., 1990). The key characteristic of asthma is reversible airflow limitation, which can occur during acute exacerbations and usually responds rapidly to bronchodilators which act on airway smooth muscle (ASM).

One of the most common chronic respiratory conditions (and the focus of the present thesis) is fixed airflow limitation, known as COPD which, according to the World Health Organization, causes more than 3 million deaths across the world each year (World Health Organisation, 2012). Despite the high prevalence of COPD and the impact it has on modern society and the healthcare system (McKenzie et al., 2003), the underlying mechanisms producing many of the symptoms associated with COPD
are not well understood. A COPD classification is determined by the presence of irreversible airflow limitation (Pauwels et al., 2001), most often defined as a decrease in the volume of air that can be forcefully expired in 1 s, the FEV₁ (Mannino, 2002). The development of COPD most often results from the cumulative effects of inhaled toxic particles, particularly cigarette smoke in developed countries (Stockley et al., 2009). Patients with this condition show an accelerated decline in lung function over the time they have smoked (van den Berge et al., 2012). Pathological features of COPD include airway inflammation (Saetta et al., 1998), remodelling of small (Hogg et al., 2004) and large airways (Tiddens et al., 1995), loss of small airways (McDonough et al., 2011) and emphysema (Hogg et al., 2009). With respect to airway remodelling, gross changes are observed to the thickness of the airway wall (Patel et al., 2008) as well as changes within the layer of ASM (Kuwano et al., 1993), a structure which is critical in determining airway wall calibre and airflow.

Functional abnormalities in COPD include lung hyperinflation (O'Donnell and Laveneziana, 2006), an impaired bronchodilator response to deep inhalation (Scichilone et al., 2008) and airway hyperresponsiveness (AHR) - an increased bronchoconstrictor response to an inhaled contractile agonist (Postma and Kerstjens, 1998). AHR in COPD may be due to underlying factors such as inflammation, remodelling of the airway wall, dissociation of the airway wall from the recoil effects of the lung, reduced lung recoil and / or abnormalities of the ASM itself. Notably, AHR is defined by an exaggerated percent fall in baseline lung function following inhalation of a bronchoconstrictor agent. Since baseline lung function is reduced in subjects with COPD (by definition), the percent change may in fact represent only a small absolute change (Jones et al., 2016a) so that some of the apparent AHR in COPD is actually an artefact of measurement. The significance of AHR in COPD therefore remains unclear.

The following review of the literature provides a broad and then a more in-depth overview of COPD, from clinical assessment and severity to the underlying pathology. Key structural and mechanical
changes in subjects with COPD will be described, providing the necessary background to support the experiments conducted in this thesis. Broadly speaking, a series of physiological experiments were performed to better understand how structural and mechanical changes to the large and small airways contribute to the onset of fixed airflow limitation, with a focus on AHR and bronchodilator response to deep inspiration (DI).

1.2 Chronic obstructive pulmonary disease (COPD)

A diagnosis or classification of COPD is characterised by irreversible airflow limitation and this is demonstrated by a progressive decline in lung function (Scichilone et al., 2006, Woolcock et al., 1991, Fletcher, 1976, Donaldson et al., 2002) compared with people with normal lung function. The degree of airflow limitation is related to the severity of the disease and is associated with the cumulative effect of inhaled noxious gas particles, mostly due to cigarette smoking (Stockley et al., 2009, Celli et al., 2004b). This disease is a predominantly smoking-related disease that becomes clinically apparent later in life, with symptoms that include chronic cough, wheeze, breathlessness and increased sputum production (Mannino, 2002, Mannino, 2003). Characteristics of COPD include hyperinflation of the lungs (O'Donnell and Laveneziana, 2006), an impaired response to deep inhalation (Scichilone et al., 2004) as well as AHR (Postma and Kerstjens, 1998, Barnes, 2006). Many other risk factors for the development of COPD other than smoking have been suggested which include genetic factors, environmental exposures, the burning of biomass fuels for cooking and heating (especially in the developing countries) (Gnatiuc and Caramori, 2014) and air pollution (Mannino and Buist, 2007).

Prevalence and severity of COPD

The prevalence of COPD is difficult to assess but a study by Mannino et al. found that COPD occurs in about 7% of former smokers, 3% in non-smokers and 14% of current smokers (Mannino et al., 2000). It has been estimated that 14.5% of Australians over the age of 40 have some form of airflow
limitation and that this figure increases to 29.2% in people aged over 75 years (Toelle et al., 2013). According to the World Health Organization, COPD was the 5th leading cause of deaths in 2013 with fatalities expected to increase by more than 30% in the next 10 years. In 2014, approximately 7000 deaths in Australia were attributed to COPD (including emphysema) which, if current trends continue, would make COPD-related deaths the third leading cause of death worldwide by 2030 (World Health Organisation, 2012). Current rules for reporting cause of death typically only provide the immediate primary cause and not the underlying cause and for this reason mortality associated with COPD may be underestimated / under-reported in the general population (ACAM, 2011). Several other diseases occur as co-morbidities with COPD, such as pulmonary hypertension (Chaouat et al., 2005), which can lead to right heart failure (MacNee, 1994) and would not be reported as a COPD-caused death. Other co-morbidities include chronic renal failure (Mapel and Marton, 2013) and gastroesophageal reflux disease (Terada et al., 2008). The current burden that COPD places on the healthcare system in Australia is estimated to be around $92.9 million per year, which accounts for 1.3% of all direct expenditure on disease (Australian Institute of Health and Welfare, 2014).

Detection and classification of COPD

Assessment of COPD is typically by changes to a patient’s lung function. The most commonly used test to determine lung function is spirometry, which measures exhaled gas volumes in relation to time. Spirometry is widely used as a screening tool to measure the prevalence and severity of disease, response to treatment, progression of the disease and for clinical research. Specifically, spirometry measures the volume of air inhaled or exhaled as a function of time (Miller et al., 2005). The most commonly derived measures are the subject’s forced vital capacity (FVC) and FEV1. Firstly, the patient performs a maximum inhalation to total lung capacity (TLC - the maximum volume of gas after voluntary inhalation), which is followed by a forced expiration to residual volume (RV - the volume of gas remaining in the lungs following maximum voluntary exhalation). The test is conducted over the full range of vital capacity (VC - the greatest volume of air that can be expelled
from the lungs after taking the deepest possible breath). From the spirometry test, the most commonly derived measures are the subject’s forced vital capacity (FVC) and FEV$_1$.

In healthy subjects, approximately 80% of the FVC can be expelled in 6 s or less (Barreiro and Perillo, 2004). The FEV$_1$/FVC ratio is the proportion of the FVC that can be expired in the 1$^{st}$ s. The ratio, FEV$_1$/FVC, is a clinically useful index of airflow imitation and is effectively a measure of the effectiveness of the lungs to empty. The parameters FEV$_1$ and FVC depend upon both the ability of the lungs to change volume and to generate airflow, therefore allowing relatively simple volume measurements to track both restrictive (volume change / compliance) and obstructive (airflow / resistance) defects of the respiratory system (Table 1.1).

**Table 1.1: Classification of obstructive vs restrictive diseases using spirometry.**

<table>
<thead>
<tr>
<th>Types of disease</th>
<th>FEV$_1$</th>
<th>FVC</th>
<th>FEV$_1$/FVC</th>
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<tr>
<td>Obstructive</td>
<td>Reduced</td>
<td>Normal to reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Emphysema, bronchiectasis, bronchiolitis, cystic fibrosis, asthma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Restrictive</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Normal to increased</td>
</tr>
<tr>
<td>Pulmonary fibrosis, asbestosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Normal to reduced</td>
</tr>
<tr>
<td>Asbestosis in heavy smokers, bronchiectasis, pulmonary fibrosis and emphysema</td>
<td></td>
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Spirometry outputs are often displayed as flow-volume loops (Figure 1.1). Restrictive diseases such as pulmonary fibrosis present flow-volume loops which are qualitatively similar to healthy people (Figure 1.1A) but with reduced volumes and flows (Egan et al., 2005). In contrast, obstructive disease causes a scalloping of the expiratory limb of the flow-volume loop (Figure 1.1B), that is, the loop is concave along its expiratory portion (Mead, 1978).
Figure 1.1: Example traces of flow-volume loops. A) a normal, healthy subject and; B) a subject classified as having chronic obstructive pulmonary disease (COPD). In the figure, expiratory flow is positive and inspiratory flow is negative, while the x-axis represents an increasing expiratory volume from total lung capacity to residual volume RV (i.e., forced vital capacity (FVC)). Note that COPD causes a reduction in volume change, FVC, airflow limitation and a characteristic scalloped-shape to the expiratory limb (Miller et al., 2005).

Spirometry is often performed before and after the administration of a bronchodilator. Short-acting β2-agonists, such as salbutamol (100 μg/puff), relaxes the ASM, reverses obstruction and facilitates greater airflow in asthmatics, but has little effect on patients diagnosed with COPD (Toelle et al., 2013). A positive bronchodilator response (increase in FEV₁ > 15% and > 200 mL compared with pre-bronchodilator measurements) is commonly used to distinguish between fixed (i.e., COPD) and reversible (i.e., asthma) airflow limitation (Pellegrino et al., 2005). The inability of bronchodilators to improve airflow is one of the most definitive characteristics of COPD (Toelle et al., 2013).

Since patients diagnosed with COPD respond minimally to bronchodilators, it might be reasonable to suspect that the obstruction is not exacerbated by bronchoconstrictors, in contrast to patients with asthma where AHR is a fundamental characteristic of the disease (O'Byrne, 1986). The excessive bronchoconstrictor response to external stimuli is normally measured using a dose-response curve
from which the sensitivity (left-ward shift in the dose-response curve), reactivity (slope of the dose-response curve) and maximal response can be deduced (Moreno et al., 1986, Woolcock et al., 1984). Sensitivity to the inhaled bronchoconstrictor is the dose that elicits a given response (e.g., the threshold dose, the dose that produces a 20% change from baseline). In healthy subjects, a maximum response to bronchoconstrictor drugs is normally reached where increasing doses no longer cause any additional bronchoconstriction (Postma and Kerstjens, 1998, Woolcock et al., 1984). In contrast, patients diagnosed with asthma are often so responsive that a maximum response cannot be safely reached. Patients with asthma show an increase in both the sensitivity, reactivity and the maximum response to various contractile agonists such as methacholine (Brusasco et al., 1999, Juniper et al., 1978, Skloot et al., 1995) and histamine (Juniper et al., 1978, Cockcroft et al., 1977, Woolcock et al., 1984). In COPD, the maximum or plateau response to bronchoconstrictors is increased compared with healthy subjects, but not to the extent seen in asthma (Du Toit et al., 1986). Similarly, patients with COPD show increased sensitivity to inhaled bronchoconstrictors, but the increased sensitivity is much less than seen in typical asthma patients (Woolcock et al., 1984). Therefore, while AHR is common in patients diagnosed with COPD (Postma and Kerstjens, 1998), the degree of increased sensitivity and maximal response is much less than in asthma, which raises the question as to whether AHR is an important component in COPD.

It is also important to acknowledge the potential effect or normalisation when reporting the presence and severity of AHR. Changes in response to a bronchoconstrictor agonist are expressed as a proportion of FEV\textsubscript{1}. Bronchoconstrictor response will therefore appear larger in subjects with COPD since lung function is reduced and a small change will appear proportionally greater. In a review by Jones et al. (Jones et al., 2016a), baseline values and the changes in lung function during a bronchial constriction test were expressed as absolute values (mL and not %) and the results of the analysis indicated that the absolute change could be quite low in subjects with COPD, even less than subjects without disease (Figure 1.2). The authors concluded that not only are the airways in COPD unable to
open, they are also resistant to “narrowing” – they seem to behave as semi-rigid structures. For these reasons, the significance of AHR in COPD is an unresolved area of research.

Figure 1.2: Schematic of dose-responses from human subjects. A subject with chronic obstructive pulmonary disease (COPD), mild and moderate asthma are compared with a control subject. Rather than expressing response to agonist as a percentage of FEV\textsubscript{1}, data was reported as absolute change (Δ). Moderate asthma exhibits a greater airway responsiveness than mild asthma or COPD. The inference is that absolute changes in COPD could be less than in individuals with normal lung function. Adapted from (Jones et al., 2016a).

Currently two common standards are used to define fixed airflow limitation. Firstly, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) standard set by the GOLD committee to provide a consistent criterion (Rabe et al., 2007, Vogelmeier et al., 2017) have defined COPD by a post-bronchodilator FEV\textsubscript{1}/FVC ratio < 0.7. Furthermore, the GOLD standard classifies airflow limitation as mild, moderate or severe based on the FEV\textsubscript{1} relative to the predicted value (Table 1.2) (Pauwels et al., 2001, Decramer et al., 2012).
Table 1.2: Severity of COPD based on the FEV\textsubscript{1} % predicted.

<table>
<thead>
<tr>
<th>GOLD stage</th>
<th>Severity of disease</th>
<th>FEV\textsubscript{1} %pred</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Mild</td>
<td>$\geq 80$</td>
</tr>
<tr>
<td>Stage II</td>
<td>Moderate</td>
<td>50-80</td>
</tr>
<tr>
<td>Stage III</td>
<td>Severe</td>
<td>30-50</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Very severe</td>
<td>$&lt; 30$</td>
</tr>
</tbody>
</table>

Sub-classification of chronic obstructive pulmonary disease (COPD) as mild, moderate, severe and very severe based on the FEV\textsubscript{1} relative to the predicted value (Decramer et al., 2012, Pauwels et al., 2001).

Even though the GOLD standard is widely used in the clinical setting, there are limitations to the criteria. Lung function, including the FEV\textsubscript{1}/FVC ratio is known to fall in the elderly and although the predicted population values used in the GOLD standard are corrected for age, the fixed cut-off of FEV\textsubscript{1}/FVC \textless{} 0.7 is not corrected for age or sex. Not taking into account the sex or age of the patient could lead to over-diagnosis of COPD, especially in elderly people. The main alternative to the GOLD classification is the lower limit of normal (LLN), which is equal to the 5\textsuperscript{th} percentile of a healthy non-smoking population, that is an FEV\textsubscript{1} less than 95\% of the normal population, taking into consideration the age, sex and height matched population (McCarthy, 2012). A subject would be classified as having COPD if both the FEV\textsubscript{1} and FEV\textsubscript{1}/FVC were below the LLN, i.e., in the bottom 5\% of the normal population.

Although both the GOLD and LLN classifications are widely used, studies have conflicting reports on which technique is more suitable in defining COPD (Guder et al., 2012, Karrasch et al., 2016). Using the LLN technique may actually under-diagnose COPD, particularly in younger patients who are more likely to have an FEV\textsubscript{1}/FVC > 0.7 (Mohamed Hoesein et al., 2011, Colak et al., 2013). For example, a study conducted by Calverley et al. showed that using the LLN classification in place of the GOLD standard would have excluded patients with significant morbidity from the diagnosis of COPD in the TIOtropium Safety and Performance In Respimat® study (Calverley et al., 2018). There
is also an ongoing debate whether only measuring fixed airflow limitation alone is sufficient to confirm a diagnosis of clinical COPD, with many proposing alternative measurements (Schermer et al., 2016, Quanjer et al., 2014) as briefly discussed below.

Due to the difficulty in assessing COPD from fixed airflow limitation alone, other methods are also used in the assessment of fixed airflow limitation and airway disease. A common approach to assessing the severity of COPD is through exercise testing (Oga et al., 2003, Celli et al., 2004a) as patients suffer from breathlessness especially upon exertion (van der Molen et al., 2003). Other more specific tests for disease and functional abnormalities associated with cigarette smoking and COPD include the diffusion capacity of the lung for carbon monoxide (DLCO) (Yoshimi and Seyama, 2007) and lung volume measurements to assess hyperinflation (Enright and Kaminsky, 2003). Measurements of DLCO are easy to determine, with DLCO values normally reduced in patients with COPD (Boulet et al., 1998, Fabbri et al., 2003, Magnussen et al., 1998, Hogg et al., 1994), likely due to loss of alveoli (Sciurba, 2004). Lung hyperinflation is another characteristic feature of COPD (Mergoni and Rossi, 2001, Sciurba, 2004, O'Donnell and Laveneziana, 2006), evident as an elevated RV and FRC which is the amount of gas remaining in the lungs after a normal expiration during quiet breathing. Both of these measurements can be measured using plethysmography, or by using nitrogen washout techniques (Rabe et al., 2007).

*Treatment of COPD*

While there is no cure for COPD, smoking cessation improves the quality of life, reduces the frequency of exacerbations and slows the rate of decline in lung function (Anthonisen et al., 1994, Decramer et al., 2005). Nicotine replacement therapies (Stead et al., 2012, Stead and Hughes, 2012) are available to help with smoking cessation, but it may take years before any smoking cessation programs have an impact on mortality rates due to COPD (Feenstra et al., 2001). Other treatments available include pulmonary rehabilitation programs (Johnston and Grimmer-Somers, 2010,
Wehrmeister *et al.*, 2011) which focuses on the patient’s physical and emotional well-being and is associated with improved quality of life, increased exercise tolerance and reduced exacerbations. Fixed combination therapy of long-acting bronchodilators and inhaled corticosteroids (Calverley *et al.*, 2003, Rabe *et al.*, 2007, Vestbo *et al.*, 2017, Singh *et al.*, 2016) and therapy with long-acting anti-cholinergics (Tashkin *et al.*, 2008) have been shown to improve outcomes (lung function, symptoms, quality of life, exacerbations) in patients with COPD. Combination therapies with long-acting bronchodilators and long-acting anti-cholinergics also produce some improvement in FEV₁ in the range of ~150-200 mL (Vogelmeier *et al.*, 2013, Buhl *et al.*, 2015), but the overall improvement in the FEV₁/FVC ratio is only approximately 5%.

Since pharmacotherapy treatments used to date are predominantly derived from those used to treat reversible airflow limitation (i.e., asthma), and, given that COPD is defined as irreversible airflow limitation, it is not surprising that current pharmacological treatments are only marginally effective in the management of COPD. The absence of a response to treatment suggests a fundamentally different pathophysiology of COPD compared with asthma.

1.3 Anatomical / histological changes in COPD

There are three main pathological features of COPD that are evident in imaging, histology and post-mortem examination. Firstly, changes in the lung parenchyma with destruction and enlargement of the alveolar spaces, called emphysema (Hogg, 2004a, James and Wenzel, 2007, Cosio Piqueras and Cosio, 2001, Barnes, 2008). Secondly, inflammation and destruction of the small airways referred to as bronchiolitis (Hogg *et al.*, 2004) and finally, there is extensive inflammation of the conducting airways with daily cough and/or mucus production known as chronic bronchitis (Saetta *et al.*, 1997). These inflammatory changes (as seen in Figure 1.3B) are observed together with remodelling of the airway wall with changes to the epithelium, mucous glands, submucosa and ASM (Hogg *et al.*, 2004) compared with a healthy airway (Figure 1.3A).
Figure 1.3: Airway pathology in COPD: A) an airway (arrow) adjacent to a blood vessel from a normal healthy individual. Note the thin wall relative to the airway lumen; B) an airway from a smoker with fixed airflow limitation. The airway wall is thickened and lumen is narrower, containing mucus and inflammatory cells within and around the airways. Adapted from (Baraldo et al., 2012).

Emphysema

Anatomically, emphysema is seen as the destruction of the alveoli and enlargement of the airspaces (Snider, 1979). From imaging studies using computed tomography (CT), two forms of emphysema are observed - centrilobular and panacinar - defined by the region of the acinus (i.e., the alveolar sacs distal to a single respiratory bronchiole) that is destroyed (Kranenburg et al., 2005). Centrilobular emphysema is observed as focal destruction confined to the respiratory bronchioles and the central parts of the acinus (Figure 1.4). It is associated with smoking (Kim et al., 1991, Finkelstein et al., 1995) and is more often found in the upper lobes. Panacinar emphysema is characterised as the uniform dilation of airspaces from the respiratory bronchioles to the alveoli (Thurlbeck, 1995) mostly found in the lower lung lobes (Hogg, 2004b), and is associated with loss of elastic recoil and higher lung compliance (Kim et al., 1991, Finkelstein et al., 1995).
Figure 1.4: Gross appearance of a resected lung showing centrilobular emphysema. Note the location of the emphysema predominantly in the upper aspects of the lobes (Jeffery, 1998).

**Obstructive bronchiolitis**

In the smaller peripheral airways, both the destruction and inflammation of the bronchioles (McDonough et al., 2011) are observed, similar to the loss of parenchyma. This combination of airway loss and inflammation is referred to as obstructive bronchiolitis (Hogg et al., 1968, Matsuba and Thurlbeck, 1972). The most pronounced feature of bronchiolitis is the loss of small airways, causing a reduction in gas exchange and a reduction to airflow (Scott et al., 1997). Loss of terminal bronchioles in bronchiolitis is related to the severity of COPD symptoms (Hogg et al., 1968, Nagai et al., 1985a, Wright et al., 1983, McDonough et al., 2011). The loss of terminal bronchioles has been shown to occur in the non-emphysematous part of the lung suggesting that small airway loss occurs prior to the onset of emphysematous lung destruction. Another significant feature of obstructive bronchiolitis is inflammation, which is associated with an increased production of mucus (ten Hacken et al., 1999) and neutrophil and mast cells infiltration (Battaglia et al., 2007). The increased production of mucus is associated with goblet cell metaplasia and contributes to the occlusion of the lumen in peripheral airways (Macklem et al., 1970).
The pathology of small airways and the degree of inflammation present in obstructive bronchiolitis is thought to be due to the exposure to cigarette smoking and occurs earlier than parenchymal destruction. Inflammation in the small airways can be detected relatively early (i.e., well before 30 years of age) (Buist et al., 1979, Nemery et al., 1981). The study of young smokers by Niewoehner et al. showed existing pathological abnormalities in the small airways in the absence of symptoms (Niewoehner et al., 1974). In this study, post-mortem lungs from young (mean age of 27 years) smokers and control subjects were obtained and studied to determine the effect of smoking and the pathological changes that occur in the peripheral airways. Pathological abnormalities including obstructive bronchiolitis were observed in the peripheral airways of young smokers. Abnormalities were characterised as clusters of pigmented macrophages in the lumen, associated with oedema and epithelial hyperplasia, with no such changes observed in normal, control lungs of subjects of comparable age (Niewoehner et al., 1974). Although the inflammation of small airways is initiated by cigarette smoke, the inflammation produced persists after smoking cessation (Cosio et al., 2009, Burgel, 2011). Numerous studies suggest that pathological changes may progress in spite of smoking cessation due to persistent inflammation (Willemse et al., 2005, Hogg, 2006, Rutgers et al., 2000).

**Chronic bronchitis**

Inflammation in patients with COPD is not just limited to the small airways, but is present in the large airways as well (Mullen et al., 1985, Saetta et al., 1997). Chronic bronchitis is characterised by chronic inflammation as well as airway remodelling of large airways (> 2mm in diameter) (Tiddens et al., 1995). Macrophages, CD8+ T lymphocytes and neutrophils are all observed during chronic bronchitis exacerbations (Saetta et al., 1999, Saetta et al., 1993, Di Stefano et al., 2004) similar to that seen in the small airways (see above). Macrophages have been shown to play a pivotal role in the pathogenesis of COPD and is classified based on their phenotype as pro-inflammatory (M1) and anti-inflammatory (M2) (Shapiro, 1999). Studies have shown that there is an increase in the number of macrophages (5-10 fold) in airways, lung parenchyma and sputum in subjects with COPD.
(Retamales et al., 2001). Studies on large airways conducted using induced sputum or bronchial biopsies have shown that the severity of disease is associated with the number of inflammatory cells present. Neutrophil infiltration in the large airways is associated with COPD exacerbations and increased sputum production (Di Stefano et al., 2004), and the number of neutrophils in induced sputum correlates with the decrease in lung function in smokers (Stanescu et al., 1996).

As previously stated, another feature of chronic bronchitis is remodelling of the airway wall, which is made up of different compartments i.e., the epithelium, lamina propria, adventitia and ASM (Figure 1.5). Increased airway wall thickness is a prominent characteristic of airway remodelling and is due to increased volumes of each compartment of the airway wall. A study performed by Hogg et al. showed that in COPD, the epithelial thickness is increased by approximately 100% and the lamina propria with the adventitia, increased by almost 50% in GOLD stages III and IV compared with GOLD stage 0 (Hogg et al., 2004). Increased volume of mucous glands and goblet cell hyperplasia lead to excessive production of mucus (Reid, 1954, Saetta et al., 2000, Lumsden et al., 1984), while hypertrophy of mucous glands along with oedema and thickening of ASM, contribute to the bronchial wall thickening seen in COPD (Lumsden et al., 1984, Saetta et al., 2000).

![Figure 1.5: Schematic of an intra-parenchymal bronchial wall. The general structure of the airway comprises a mucosal and submucosal layer. Structural alterations to these compartments (or structures therein) is defined as ‘airway remodelling’. Schematic adapted from (Bai et al., 1994).](image-url)
The ASM layer is made up of ASM cells as well as extracellular matrix (ECM) proteins, mast cells and blood vessels (Thomson et al., 1996), with any change in these constituents responsible for an increase in the ASM layer (Jones et al., 2014). Increased thickness of the ASM layer, is observed in COPD (Hogg et al., 2004) and asthma (Kuwano et al., 1993). In asthma, the ASM cells within the ASM layer is increased in the large airways due to hyperplasia and hypertrophy of ASM cells (Ebina et al., 1990b, James et al., 2012). In contrast, patients with COPD show only a modest increase in the volume (size) of ASM cells compared with controls (Ebina et al., 1990a). The thicker ASM layer, found in COPD, may be attributed to the increase in ECM. This observation has significant implications as the contractile and non-contractile functions of the ASM are altered by its interaction with the surrounding ECM through cell-surface signalling and specific receptors (Zhang and Gunst, 2008). The ECM is comprised of fluids and a combination of proteoglycans and glycosaminoglycans that have many functions including aiding in osmotic activity, structural integrity, cellular adhesion and releasing growth factors (Thomson et al., 1996). The major components of lung ECM are collagen I, III and V, elastins, proteoglycans, fibronectin, biglycan, lumican, versican, decorin and tenascin (Parameswaran et al., 2006).

Collagen is amongst the most abundant ECM protein within the lung (Vlahovic et al., 1999) and is very important in maintaining normal lung structure with studies showing that in COPD, the collagen content (Kranenburg et al., 2006, Annoni et al., 2012, Vlahovic et al., 1999) as well as its structural organization (Tjin et al., 2014) are altered. Kranenburg et al. (Kranenburg et al., 2006) also found that the expression of collagens I, III, IV, fibronectin and laminin were increased in COPD patients. The expression of ECM proteins within the lung parenchyma is altered in COPD. The amount of elastin (Wright, 1961, Chrzanowski et al., 1980), proteoglycans (van Straaten et al., 1999) and versican (Merrilees et al., 2008) in the lung parenchyma are all reduced in patients diagnosed with COPD. Changes in the composition of the ASM layer in subjects with COPD, and its implication to function is of particular interest to this project, will be discussed further in Section 1.5.
In summary, COPD involves a range of pathological changes to the lung parenchyma, small airways and large airways, including both inflammation and structural changes. Which brings us to pose the question “what are the consequences for these pathological changes?” and “how do they explain the observed symptoms found in COPD?”. Specific effects of parenchymal disease (emphysema) and airway disease (inflammation and remodelling) will now be discussed.

1.4 Pathophysiology in COPD – role of parenchymal disease

Airflow limitation

The most prominent pathological finding in COPD is emphysema, which is defined as the destruction of alveoli (Hogg, 2004a, James and Wenzel, 2007, Cosio Piqueras and Cosio, 2001, Barnes, 2008). The obvious effect of losing the alveoli is a reduced surface area for gas exchange (Snider et al., 1986), which might explain the poor DLCO observed in COPD. A loss of surface area also causes a loss of elastic recoil of the lung and reduced tethering on the bronchi leading to airflow obstruction which characterises COPD. One approach that has been used to demonstrate the effect of emphysema on lung function is to measure the static transpulmonary pressure (P_{tp}) at different lung volumes (Pare et al., 1982). In Figure 1.6, P_{tp} is plotted against different lung volumes, indicating the contribution of emphysema to pressures exerted on lung tissue (Yanai et al., 1992). For a given lung volume, P_{tp}, or ‘elastic recoil pressure’, was reduced in the presence of emphysema.
Figure 1.6: Schematic showing pressure-volume curves of a normal lung (green line) and a lung with emphysema (red line) during inflation to total lung capacity (TLC). Transpulmonary pressure ($P_{tp}$) increases (1-3) with a concomitant increase in volume until no increase in volume occurs at higher pressures (4). Maximum $P_{tp}$ is reduced in the emphysematous lung (Suki et al., 2013).

Reduced elastic recoil pressure caused by the loss and altered mechanical properties of the lung parenchyma is the most likely mechanism through which emphysema causes air flow limitation (Finucane and Colebatch, 1969). Elastic recoil is the tendency of the lung to collapse with the loss of the elastic recoil resulting in the loss of the tethering forces on the airways, which normally hold the airways open during expiration. Loss of elastic recoil results in narrowing of the airways with higher resistance to expiratory flow (Hyatt, 1983). Simply put, the loss of tethering on the airways causes the airways to collapse, especially during expiration, when intrapleural pressure is positive (Saetta et al., 1985, Scichilone et al., 2008). Expiratory airflow limitation caused by the collapse of the airways, independent of ASM contraction, will be unaffected by the use of bronchodilators, contributing to the fixed airflow limitation which is a feature of COPD (Toelle et al., 2013). The loss of elastic recoil of the lung not only causes airflow limitation, but also contributes to hyperinflation of the lungs seen in COPD (Brusasco and Fitting, 1996).
Another method used to assess the effects of emphysema on airflow is to measure the degree of emphysema macroscopically in resected lung tissue or by CT-scans (Bergin et al., 1986) and relate it to spirometry (Hogg et al., 1994, Nakano et al., 2000, Timmins et al., 2012, Cerveri et al., 2004). In a bid to correlate the extent of emphysema scores with lung function, Hogg et al. performed baseline lung function on 407 patients with a history of smoking and macroscopically examined the lungs following lung resection surgery. Reduced FEV₁ correlated with increased scores of emphysema in these patients (Hogg et al., 1994). Another study by Nakano et al. evaluated emphysema and airway wall thickening in 114 smokers with CT-measurements and found that reduced FEV₁ also correlated with increased scores of emphysema (Nakano et al., 2000). While both studies showed a correlation between emphysema and the extent of airflow limitation, these studies use scores of emphysema based on the destroyed, resected lung, whereas the spirometry reflects the function of both intact lungs (including emphysematous and non-emphysematous portions).

**Lung hyperinflation**

In addition to airway collapse, a pronounced effect of the loss of elastic recoil is lung hyperinflation: increased lung volumes including FRC, RV and TLC, relative to normal (Thurlbeck, 1983, Pellegrino and Brusasco, 1997b). Lung volumes between RV and TLC are a balance between the inward recoil of the lung and the outward recoil of the chest wall (Anthonisen, 1986). When parenchyma is lost, the collapsing force of the lung is reduced and the unchanged expanding force from the chest wall (rib cage) produces an increase in lung volume, termed static hyperinflation (Ferguson, 2006, Pride, 2005, Thomas et al., 2013). The hyperinflation from loss of elastic recoil explains some of the increase in RV and FRC observed in COPD (Gibson, 1996, Corbin et al., 1979). Static hyperinflation is however not the only component of the hyperinflation seen in cases of COPD (Ferguson, 2006).

Dynamic hyperinflation is also a partial consequence of reduced elastic recoil, although indirectly through the dynamic collapse of airways during expiration (Olaffson and Hyatt, 1969). During
Inspiration airways are held open by the positive transmural pressure ($P_{tm}$ pressure across the airway wall) and increasing lung volume reduces resistance as the airways increase in size with the rest of the lung (O'Donnell, 2001). During expiration, however, lung resistance increases due to the reduction in lung and airway size during the breath (Aldrich et al., 1989) and the pressure gradient across the airway wall reverses with a compressive force applied to the bronchi. Loss of elastic recoil greatly reduces the force holding the airways open during expiration causing significantly more airflow limitation during expiration than inspiration. Expiratory airflow limitation due to airway narrowing further increases gas trapping within the lungs (Figure 1.7) due to an imbalance between inhaled and exhaled volumes (Ferguson, 2006, Brusasco and Fitting, 1996, Pellegrino and Brusasco, 1997a, O'Donnell D, 2008) and thereby contributes to hyperinflation.

![Diagram of gas trapping during expiration in COPD](image)

**Figure 1.7: Schematic representation of gas trapping during expiration in a subject diagnosed with COPD.** A) in a normal, healthy subject, elastic recoil pressure of the lungs ($P_L$) keeps airways open and normal breathing occurs; B) in a subject with COPD, the loss of $P_L$ reduces the force that keeps airways open, allowing air to be trapped in the lungs before complete exhalation. As the subject takes another breath in, gas is trapped inside the lungs leading to hyperinflation. Schematic adapted from (Thomas et al., 2013).
Impairment in response to deep inspiration

In addition to hyperinflation and impaired gas exchange, patients with COPD may also demonstrate an impaired bronchodilatory response to deep inspiration (DI) (Crimi et al., 2002, Scichilone et al., 2008). In healthy people, taking a deep breath in, a DI causes bronchodilation that persists for several minutes (Salome et al., 2003) (Figure 1.8). The bronchodilation to DI may be reduced or absent in both patients with asthma and those with COPD (Scichilone et al., 2004, Skloot and Togias, 2003). The mechanism(s) responsible for the beneficial effects of DI is not completely understood but might include activation of neural pathways (Kesler and Canning, 1999, Skloot and Togias, 2003, Scichilone et al., 2001), relative hysteresis of the airways vs parenchyma (Froeb and Mead, 1968) or a direct effect of stretch on the ASM layer (Fredberg et al., 1997, Gunst and Wu, 2001).

Figure 1.8: Effect of deep inspiration in a healthy subject. As the subject takes a deep breath in, lung volume increases (A) and resistance (Rrs) is reduced (B), indicating that bronchodilation had occurred (Hulme et al., 2013).

Inhibition of cholinergic (Scichilone et al., 2001) or other excitatory neural pathways (Hida et al., 1984) by lung or airway stretch has been proposed as a mechanism for DI-induced bronchodilation.
Airway stretch could activate processes that reduces bronchoconstriction by causing a release of muscle relaxants such as nitric oxide (Bannenberg and Gustafsson, 1997) or cyclooxygenase products (Gao and Vanhoutte, 1993). Airway calibre, following a transient stretch, will be determined by the balance between airway and parenchymal hysteresis (Froeb and Mead, 1968, Ingram, 1990) where, if the hysteresis of the airways predominates over the parenchyma, a DI produces bronchodilation. A DI could also exert its effect by a direct effect of stretch on ASM. Several studies using isolated muscle as well as whole bronchial tubes have shown that cyclical stretch of the ASM produces bronchodilation and causes a decrease in force production (Shen et al., 1997, Fredberg et al., 1997, Noble et al., 2011). We have previously shown that the human airway wall responds to mechanical stretch with reduced contraction (Noble et al., 2011).

In two separate studies performed by Scichilone et al. (Scichilone et al., 2005) and Corsico et al. (Corsico et al., 2003), it was suggested that reduced alveolar attachments to the airways of patients with COPD could impair the bronchodilator response to DI. They speculated that the destruction of the alveolar attachments reduces the effectiveness of the distending forces so that a DI would not be able to stretch the airways that have narrowed. During a DI, radial tension is applied to the outer airway wall due to the forces of interdependence between the airways and surrounding parenchyma (Fairshter, 1985). The bronchodilator response to DI could become impaired if airways were uncoupled from the lung parenchyma (i.e., parenchymal tethering) (Suki and Bates, 2011, Lauzon et al., 2012). Hence in COPD, loss of alveolar attachments results in uncoupling of the airway from the parenchyma leading to less strain imposed on the airways by the parenchyma during a DI (Lambert and Pare, 1997). Scichilone et al. also suggested that when airways are uncoupled from surrounding parenchyma, the airway response to DI might transition from one of bronchodilation, to bronchoconstriction (Scichilone et al., 2004), perhaps due to loss of recoil pressure via parenchymal hysteresis.
An alternative theoretical mechanism that may reduce stretch of the ASM and therefore bronchodilation to DI is lung hyperinflation, commonly observed in subjects with moderate to severe COPD (O’Donnell and Laveneziana, 2006, Sciurba, 2004, Scichilone et al., 2008, Martinez et al., 1990, Decramer, 1989). Dynamic hyperinflation, and not static hyperinflation (see page 37), may cause airways to operate at a higher $P_{tm}$ and therefore at a stiffer region of their pressure-volume curve, reducing the change in ASM stretch produced by changes in $P_{tm}$ during a DI. Importantly, pressure-volumes curves of airways are non-linear and more compliant at low compared with high $P_{tm}$ (i.e., normal FRC). That is, due to the non-linear pressure-volume relationship of the airways (Harris, 2005, Venegas et al., 1998) airways become stiffer as lung volume increases during inspiration and therefore more resistant to mechanical stretch. It is therefore possible that under conditions of hyperinflation, the airway wall (including the ASM) is not sufficiently stretched to elicit mechanisms producing bronchodilation in the normal airway.

Several studies support an association between hyperinflation and reduced response to DI. In a study by Scichilone et al., using a high resolution computed tomography (HRCT)-based index of parenchymal density, it was shown that functional and radiological descriptors of air trapping and lung hyperinflation correlated with reduced or absent DI-induced bronchodilation (Scichilone et al., 2008). Increased hyperinflation and reduced lung density predicted a reduction in the bronchodilator effect of DI. An additional consideration is the capacity for respiratory muscles to generate sufficient $P_{tp}$ to distend airway tissues. In the hyperinflated state, the ability to generate high $P_{tp}$ is severely decreased leading to less radial traction of the airways, reducing the effect of DI. Salerno et al. confirmed that the effect of failure to expand the lungs due to hyperinflation is one determinant of a reduced bronchodilator response to DI (Salerno et al., 2005).
1.5 Role of airway disease in COPD

Airflow limitation

It is not just emphysema that causes obstruction in COPD as there are structural abnormalities in both the large (Tiddens et al., 1995, Patel et al., 2008) and small airways (Hogg et al., 2004). Numerous studies have shown a correlation between impaired lung function and lumen dimensions of the airways in COPD. In general, there is a decrease in the airway diameter or an increase in the number of airways with reduced diameters compared with healthy control subjects (Hogg et al., 2004, Nagai et al., 1985b, Cosio et al., 1978). These studies also show a correlation between parenchymal disease and airway disease in COPD and demonstrate that the obstruction and flow limitation seen is not due to the effects of emphysema alone.

Remodelling in small airways is a determinant of poor lung function in COPD. Findings of seminal studies (Hogg et al., 2004) show that in small airways, increased thickness of the airway wall and increased production of mucus correlates with expiratory airflow limitation in COPD, with a correlation observed between the number of inflammatory cells (neutrophils, macrophages, T-cells and B-cells) in small airways with severity of airflow limitation (Hogg et al., 2004). Other studies have shown that the number of neutrophils and mast cells were higher in small airways compared with large airways (Battaglia et al., 2007) and Saetta et al. reported that CD8+ T-cells are increased in small airways from patients with COPD and their number correlated inversely with lung function (Saetta et al., 1998). Significant increases (~20% on average) in the area of the ASM were also reported in the same study (Saetta et al., 1998). Therefore, the evidence suggests that both airway inflammation and remodelling correlate well with the airflow limitation seen in patients with COPD.

The role of large airways in the pathophysiology of COPD is less appreciated but is certainly of significance in some patients. Patel et al. used CT to show that wall thickness of airways with a lumen perimeter of 10 and 20 mm correlated independently (of emphysema score) with airflow limitation.
in 3,096 patients (Patel et al., 2008). The impact of airway wall remodelling on lung function has also been assessed by measuring dimensions of airways obtained after lung resection surgery. In a study on large cartilaginous airways from 72 patients, there was an inverse association between inner wall thickness and FEV₁/FVC (Tiddens et al., 1995). These studies reinforce the importance of the large airway in the pathogenesis of COPD.

In addition to gross airway remodelling, changes within the ASM layer are demonstrated in subjects with COPD and/or are related with subject lung function. In contrast to asthma, where hypertrophy and hyperplasia cause increased ASM thickness (James et al., 2012) little hypertrophy (Ebina et al., 1990a) with no hyperplasia of ASM cells is found in COPD. Instead, there is an inverse relationship between the volume fraction of ECM and the post-bronchodilator FEV₁ measured in patients with COPD (Jones et al., 2016b) (Figure 1.9). That is, an increased volume fraction of ECM (V_{ECM}) in the ASM layer is associated with reduced and fixed lung function. Similar inverse correlations have been shown between collagen deposition in the basement membrane or laminin expression in the ASM layer and FEV₁ %predicted (Kranenburg et al., 2006). Black et al. found that the volume fractions of elastin in both airways and alveoli were reduced in COPD patients. The authors also demonstrated a correlation between the amount of elastic fibres in the alveoli and airways and FEV₁ %predicted (Black et al., 2008). Changes in ECM proteins are therefore associated with reduced lung function, although the mechanistic link remains to be determined.
Figure 1.9: **Relationship between post-bronchodilator lung function and volume fraction of extracellular matrix (V\textsubscript{VECM}) within the airway smooth muscle layer.** Subject FEV\textsubscript{1} %pred was inversely related to V\textsubscript{VECM} in both smokers (current and ex; closed symbols), as well as never-smokers (open symbols) (Jones et al., 2016b).

**Airway hyper-responsiveness**

A common feature of COPD is AHR, i.e., increased airway narrowing in response to a bronchoconstrictor stimulus (O’Byrne, 1986). As discussed earlier, (Section 1.2), AHR is one of the hallmark features of asthma (Wiggs et al., 1990) and to a lesser extent in COPD (Postma and Kerstjens, 1998). A study by the Lung Health Study Research Group found that AHR was prevalent in ~60% of COPD patients, although it should be noted that the COPD subjects in that study only had mild / early COPD (Tashkin et al., 1992). The underlying mechanism(s) for AHR in COPD is not properly understood and a number of theories have been proposed. These include failure of DI-induced bronchodilation, loss of after-load caused by emphysema, reduced airway calibre from invagination of the wall into the lumen or airway remodelling from bronchitis leading to increased ASM force. Each will be discussed in detail below.
A possible mechanism for AHR could be due to the loss of the inhibitory (bronchodilator) effect of DI. In healthy individuals, DI produces bronchodilation (Fish et al., 1981) and the failure of DI-induced bronchodilation would result in increased contraction of the ASM and contribute to AHR if DI is important in regulating normal muscle tone (Noble et al., 2012). The potential impact of lung hyperinflation and emphysema in reducing the response to DI was discussed previously (Section 1.4). However, the increased airway wall thickness in patients with COPD (Patel et al., 2008, Tiddens et al., 1995) could also make airways stiffer compared to those of healthy people. Increased airway stiffness potentially limits airway wall stretch during a deep breath independent of emphysema (Noble et al., 2007). In asthma, despite the absence of emphysema, the bronchodilator response to DI is also impaired (Brusasco et al., 1999, Fish et al., 1981). This suggests that airway disease alone without emphysema can cause a loss of the bronchodilator response to DI, possibly as a result of airway wall remodelling.

Whether the airway wall is actually stiffer in COPD is not well established. Evidence for increased airway stiffness in COPD is derived from a parameter referred to as ‘distensibility’. In broad terms, distensibility describes the capacity of the airway lumen to expand during an arbitrarily defined inflationary manoeuvre such as a tidal breath or DI (Johns et al., 2000). Data from HRCT demonstrates a decrease in the distensibility of large to medium sized airways in subjects with GOLD stage II and IV COPD (see Table 1.2) (Diaz et al., 2012). Similarly, assessment of airway distensibility using oscillatory mechanics demonstrates that airways are less distensible in subjects with moderate to severe COPD compared with normal controls (Baldi et al., 2010). Further studies are required to determine if airway remodelling increases airway stiffness in COPD, whether this contributes to a loss of response to DI and in turn AHR.

The magnitude of ASM shortening is balanced by afterload - when an afterload is applied to the muscle, shortening is decreased. Experiments with human isolated ASM (Ishida et al., 1992,
Schellenberg *et al.*, 1991, Opazo Saez *et al.*, 2002) show that increasing the elastic loads on the ASM decreased shortening capacity. In cardiac, skeletal and smooth muscle, as the muscle shortens, the force it can generate decreases, as its position on the length-tension curve is altered (Wang *et al.*, 2001) If the afterload is reduced, then the muscle can still generate enough force to cause shortening at very small muscle lengths i.e., more total shortening.

In ASM, however, less shortening is observed than expected from the muscle’s length-tension properties. The reduced shortening capacity of ASM is referred to as shortening inhibition (Gunst and Fredberg, 2003) that is likely generated by slowly cycling cross-bridges in the late phase of smooth muscle contraction. Crossbridge cycling rate is not constant in ASM, with rapid cycling in the first few second of contraction transitioning to slowly cycling crossbridges late in contraction (Stephens *et al.*, 1986). In the presence of a large afterload, shortening velocity of ASM declines when afterload is increased (Gunst *et al.*, 1993), reducing the amount of shortening that occurs early in contraction when the crossbridges are more actively cycling. As time passes, quite rapidly during ASM contraction, cross-bridges stop cycling and enter into a slowly cycling or “latch” state (Hai and Murphy, 1988b, Hai and Murphy, 1988a), a mechanism of ASM mechanics which is still poorly understood. The non-cycling bridges put a halt on tension development and hold the muscle in position, preventing muscle shortening and because of the slow shortening velocity early in contraction (from increased afterload), relatively less shortening occurs.

Elastic after-loads opposing airway narrowing are potentially reduced in COPD and if so will contribute to AHR. During normal contraction in healthy airways, the ASM must stretch the parenchyma which is attached to the airway as well as narrow the airway itself (Lambert and Pare, 1997). The normal elastic recoil of the lung provides an afterload which opposes forces that narrow the airway. Emphysema, where present, will be associated with reduced elastic recoil of the lung.
(Finucane and Colebatch, 1969). With reduced elastic recoil, the shortening capacity of the ASM and narrowing capacity of the airway is therefore increased.

The capacity of the ASM to narrow the airways can also be increased by geometric factors associated with remodelling of the airways (Moreno et al., 1986). James et al. used a mathematical model to show that the same degree of ASM shortening in a thickened airway wall would increase the amount of airway narrowing compared with normal, healthy airways (James et al., 1989). Wall thickening amplifies airway narrowing because the increase in wall volume internal to the ASM encroaches onto the lumen causing more airflow limitation (James et al., 1989, Wiggs et al., 1992). Thickening of the airway wall serosal to the muscle layer could also uncouple the ASM from parenchymal afterloads (Lambert et al., 1993). However, in a study conducted by Niimi et al., a negative correlation was observed between airway wall thickness and AHR in asthmatics suggesting that the predicted geometric factors are weaker than expected or possibly that a thicker airway wall is stiffer and adds significant afterload, limiting ASM shortening. The authors speculated that it is not just the forces leading to airway contraction that are important, but also the mechanical properties of the airway wall itself (Niimi et al., 2003).

As discussed previously, in asthma, the thickness of the ASM layer is increased due to both hypertrophy and hyperplasia of ASM cells (James et al., 2012). Various mathematical models have shown a correlation between ASM mass and narrowing of the airways (Lambert et al., 1993, Affonce and Lutchen, 2006), as an increased amount of ASM leads to an increase in the total force production generated by the muscle, allowing it to overcome the physiological loads opposing ASM shortening. Our group have found that in human bronchi an increase in the amount of ASM correlated with force production by ASM and airway narrowing (Noble et al., 2013). The logical conclusion was that increased force production increases the capacity of the airways to narrow for a given afterload. Hypertrophy and/or hyperplasia of ASM cells should then increase ASM force and shortening since
both may be associated with a greater density of ASM contractile units. However, no hyperplasia and only modest hypertrophy of ASM have been found in the airways of COPD patients to date (Ebina et al., 1990a).

An increase in force production by ASM could also result from a change in the ASM phenotype and an increase in the intrinsic contractility of the muscle. Increased contractility can be defined as the increase in maximum force or shortening of a muscle at the same preloads and afterloads for the same amount of muscle. While the number of studies examining ASM contractility in COPD are far less than in asthma, there is evidence that the mechanical phenotype of ASM is different in subjects with COPD. Opazo Saez et al. used peripheral bronchial rings from subjects with COPD to show an increase in ASM contractility. The isometric stress (force per ASM area) produced by ASM, in response to acetylcholine, was negatively correlated with baseline lung function, confirming that a phenotype change is present in COPD (Opazo Saez et al., 2000). Increased airway narrowing and AHR in COPD patients could then be a result of increased force production from a change in ASM contractility associated with airway inflammation and remodelling.

It is unclear whether the above change in ASM phenotype (established by mechanical performance) is related to changes in the composition of the ASM layer, that is, an increase in the volume fraction of ECM. Transmission of force throughout the ASM layer is mediated by cell to matrix connections, via transmembrane proteins known as integrins (Zhang and Gunst, 2008). If an increase in V_{ECM} within the ASM layer results in greater ASM-ECM coupling, this could theoretically improve the transmission of force from the ASM cells to the rest of the airway wall leading to increased narrowing for the same level of ASM cell activation. Alternatively, a thicker and potentially stiffer ECM could inhibit narrowing by restricting ASM shortening via increased ASM loads within the smooth muscle layer (Meiss, 1999, Pare et al., 2007). Changes in ECM within the ASM layer could theoretically
increase or decrease ASM force and shortening and therefore its significance to airway narrowing (AHR) has yet to be tested.

Traditionally, a change in ASM phenotype or contractility has been thought of as a slow change caused by persistent inflammation. However, ASM has been shown to rapidly modify its cytoskeleton and adapt to applied loads or resting length. This plasticity of ASM could provide a mechanism for altered ASM contractility (Pratusevich et al., 1995, Seow, 2005). Smooth muscle shows plasticity of their length-tension curves, called length-adaptation, which allows the muscle to develop the same amount of force over a large range of muscle lengths (Bai et al., 2004, Wang et al., 2000, Wang et al., 2001) after a period of adaption to the new resting length. The process of length-adaptation, is triggered by re-arrangement of the contractile apparatus (Bosse et al., 2008, Seow, 2005) and the proteins ensuring linkage between the cells within the tissue and the ECM (Gunst et al., 1995, Zhang and Gunst, 2008). A loss of elastic recoil which causes the airways to collapse, or thickening of the outer airway wall (serosal to the ASM) would allow passive shortening of the ASM, which would tend to reduce the narrowing and force generation by the muscle. However, due to length-adaptation, once the ASM adapts, the new shorter length theoretically becomes the optimum length for force production and the muscle’s force and narrowing from this length will increase (Figure 1.10). In essence, the current operating length of the ASM will become the new optimum length for force production allowing greater shortening than that expected from the initial force-length relationship (Wang et al., 2001).
Figure 1.10: Length-adaptation of airway smooth muscle (ASM). When the ASM is shortened beyond its optimum length, force production decreases (open circles). If the muscle is held at a shortened length for a prolonged period, force production increases as the muscle adapts to its new length (closed circles). Adaptive changes result in a left-ward shift in the length-tension curve. Figure adapted from (Wang et al., 2001).

Plasticity of ASM is also demonstrated through a phenomenon called “force-adaptation”, which is defined as a gain in contractile capacity induced by tone (Bosse et al., 2009, Bosse et al., 2010). That is, if ASM is kept partly contracted, the maximum force the muscle can develop increases over time. If ASM in COPD patients is normally under a higher level of resting tone, then the contractility of this muscle could increase, producing AHR. The resting contraction of ASM in the absence of an exogenous stimulus is termed ‘intrinsic tone’ and has been previously observed in isolated human ASM (Wylam et al., 2012, Rabe et al., 1993) and bronchi (Ellis and Undem, 1994). The physiological causes of intrinsic tone are varied and include changes in calcium sensitization pathways, histamine release, cholinergic stimuli, release of leukotrienes or extracellular calcium leaking into the cells (Fox and Daniel, 1979, Wylam et al., 2012). Numerous studies performed in mice in vivo and in isolated ASM tissue (Bosse et al., 2009, Bosse et al., 2010, Lee-Gosselin et al., 2015, Pascoe et al., 2012a) have shown that an increase in tone enhances the contractile capacity of the ASM. However, the lack
of response to inhaled bronchodilator in COPD argues against the possibility that ASM tone is increased, and therefore that AHR is related to force adaptation.

1.6. Aims and organization

The prior literature review describes COPD as a heterogeneous disease that is commonly associated with a progressive decline in lung function and irreversible airflow limitation. Several outstanding questions are identified relating to the origin of AHR in COPD, mechanism for disruption of the bronchodilatory response to DI and the potential role of the composition of the ASM layer, specifically VVECM within the ASM layer. A series of physiological experiments are now presented in the form of prospective “papers for publication” (Chapters 2, 3 and 4) in preparation for eventual journal submission as well as the fourth result chapter (Chapter 5) that has been published in a peer-reviewed journal (Cairncross et al., 2018). Result chapters are designed to complement, without necessarily replicating concepts outlined in the literature review.

In Chapter 2, we measured airway narrowing capacity and the response to DI of large airway segments from subjects with and without COPD undergoing lobectomies or pneumonectomies. The aim of the study was to determine if the apparent AHR seen in COPD and disrupted response to DI was related to an abnormality of the airway wall, with a particular focus on the VVECM within the ASM layer.

In Chapter 3, we examined contractile properties of peripheral airway rings from human subjects undergoing lobectomies or pneumonectomies. The aim of the study was to determine the effect of the composition of the ASM layer on contraction to applied agonists, and also intrinsic tone in the absence of an exogenous stimulus.
In Chapter 4, *in vitro* experiments performed in Chapter 3 were translated to the *in vivo* environment, and the composition of the ASM layer was related to bronchoconstrictor response to bronchial challenge in subjects with and without COPD. The aim of the study was to determine whether airway responsiveness measured in the clinical laboratory is impacted by the composition of the ASM layer.

In Chapter 5, we tested the hypothesis that in patients presenting with hyperinflation, DI is less effective at producing bronchodilation since the airway exists at a stiffer region of the pressure-volume curve and therefore produces less mechanical stretch on the ASM. For this study, an *in vitro* model was used, comprising a porcine airway segment under conditions simulating normal inflation and hyperinflation. The aim of the study was therefore to determine how hyperinflation at the level of an airway impacts bronchodilator response to DI.

Finally, in the concluding chapter (Chapter 6), a general discussion provides a broader description of how each result chapter, in isolation and then together, advances our understanding of COPD.
Chapter 2: Airway narrowing and response to deep inspiration in bronchial segments from subjects with fixed airflow limitation.

2.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a prevalent respiratory disease and is estimated to be the third global leading cause of death by 2020 (Mannino et al., 2002). This disease is characterised by irreversible airflow obstruction and progressive exertional breathlessness (Scichilone et al., 2006, Woolcock et al., 1991, Fletcher, 1976, Donaldson et al., 2002). The underlying pathologies in subjects with COPD include emphysema (Hogg et al., 2009), loss of small airways (McDonough et al., 2011) and inflammation and remodelling in the small (Hogg et al., 2004) and large airways (Tiddens et al., 1995). Airway disease (remodelling and inflammation) predominates in some individuals, potentially determined by genetic factors in subjects exposed to noxious stimuli, most notably cigarette smoke (Patel et al., 2008).

Airway remodelling in COPD has been demonstrated by an increase in gross wall thickness using computed tomography (Nakano et al., 2000, Nakano et al., 2002), and an increase in airway smooth muscle (ASM) (Kuwano et al., 1993) and inner wall thickness determined through histopathology (Tiddens et al., 1995). More recently, it has been shown that the structural composition of the ASM layer is also altered in subjects with COPD (Jones et al., 2016b). The ASM tissue layer is composed of both extracellular matrix (ECM) proteins and the muscle cells themselves and other tissues such as blood vessels and inflammatory cells (Thomson et al., 1996). In COPD, there is an increase in the volume fraction of ECM ($V_{V_{\text{ECM}}}$) within the ASM layer in both small and large airways (Jones et al., 2016b). Compositional changes to the ASM layer in COPD are in contrast to subjects with asthma, where the increased ASM layer thickness is due to a proportional increase in all ASM layer components (James et al., 2012). The composition of the ASM layer structure may therefore represent an important structural marker distinguishing COPD and asthma.
The functional implications of increased $V_{\text{VECM}}$ within the ASM layer are not known but may impact force transmission throughout the layer. For ASM to transmit force from the cytoskeleton to surrounding tissues, the ASM cells must be anchored to the ECM (Zhang and Gunst, 2008). Similarly, the transmission of force to ASM from breathing movements such as deep inspiration (DI) depends on the connections to and within the ECM (Irons et al., 2018). Changes in ASM composition therefore, in theory, impact airway narrowing capacity and bronchodilatory response to DI (Noble et al., 2011). Notably, while airway hyper-responsiveness (AHR) is more pronounced in subjects with asthma (O'Byrne, 1986), it is frequently reported in subjects diagnosed with COPD (Postma and Kerstjens, 1998, Barnes, 2006). Subjects with COPD also exhibit a reduced bronchodilatory response to DI (Scichilone et al., 2004), which is considered an important mechanism to maintain airway patency (An et al., 2007). Bronchoconstriction and failure to dilate to DI both contribute to airflow limitation in COPD.

In view of the changes observed to the structure of the ASM layer in subjects with COPD, we reasoned that abnormalities such as AHR and reduced bronchodilation to DI could be traced back to abnormalities intrinsic to the airway wall. Subjects undergoing lung resection surgery were recruited and lung function assessed including response to DI determined from the ratio of maximal to partial expiratory flow. Bronchial segments were acquired following surgery and studied in organ bath chambers for assessment of airway narrowing capacity to contractile activation and bronchodilation to simulated DI and subsequently morphometry. Findings show that increased $V_{\text{VECM}}$ within the ASM layer in COPD is associated with a concomitant increase in airway narrowing capacity, and while bronchial segments from subjects with COPD exhibited a normal response to DI, proportion of ECM correlated with DI response in vivo suggesting a modification of airway-parenchymal interactions.
2.2 Methods

Patient recruitment and measurement of lung function

Subjects (n = 61) undergoing lung resection (predominantly for peripheral lung cancers) were recruited. All subjects provided informed consent to participate in the study and approval for the study was obtained from the Human Research Ethics Committee of Sir Charles Gairdner Hospital, Perth, Western Australia, Australia (Approval number 2007–161). Subjects completed a questionnaire regarding respiratory illness, medications and smoking history. No subject had a history of asthma.

Baseline lung function was assessed on the day of surgery using a wedge spirometer (model 570, Med Science) according to American Thoracic Society and European Respiratory Society standards (Pellegrino et al., 2005). Subjects were classified as having COPD if they had a post-bronchodilator (3 puffs of salbutamol from a metered dose inhaler, 100 μg/puff) FEV$_1$ < 80% of the predicted value and an FEV$_1$/FVC < 0.7. Reference equations for predicted values were from the 3rd National Health and Nutrition Examination Survey (Hankinson et al., 1999). Response to DI in vivo was determined by the ratio of isovolumic flows from forced expiratory efforts following maximal (M) or partial (P) inspiratory efforts i.e., the M/P ratio (Pellegrino et al., 1998, Brusasco et al., 1992). Subjects were asked to breathe normally (~1 min) and perform a partial forced expiratory manoeuvre to residual volume immediately followed by a deep breath to total lung capacity (TLC) and (without taking the mouth off from the spirometer) a maximal expiratory manoeuvre. Flow-volume curves were plotted using a PowerLab data acquisition system and LabChart Software and the ratio of maximal to partial flow rate at 40% of FVC was determined. An M/P ratio > 1 represents bronchodilation after a DI and a ratio < 1 represents bronchoconstriction after a DI (Figure 2.1).
Figure 2.1: Example trace of breathing manoeuvre performed by subjects to determine the response to deep inspiration (DI). Normal tidal breathing was followed by a partial forced expiratory manoeuvre to residual volume. This was immediately followed by maximal inspiration to total lung capacity and subsequent maximal expiration. Ratio of maximal to partial (M/P) flow rate determined at 40% of forced vital capacity. In this example, the DI triggered bronchodilation, reflected as greater flow at the same lung volume.

Bronchial segment preparation

From the 61 subjects recruited for the study, suitable tissue for in vitro examination was obtained from 18 subjects. Tissue obtained post-operatively was from different lung lobes (Figure 2.2) and macroscopically normal and away from the peripheral tumour. Bronchial segments (cartilaginous) were dissected free from parenchyma and had all side-branches ligated with surgical silk, as previously described (Noble et al., 2013, Noble et al., 2011). Proximal and distal diameters were measured by the insertion of metal rods of known size (drill bits) into the airway lumen.
Figure 2.2: Schematic of the human lung depicting the different lung lobes targeted for lobectomy.

Tissue was obtained from the right upper lobe (RUL), right middle lobe (RML), right lower lobe (RLL), left upper lobe (LUL) or the left lower lobe (LLL) following lung resection surgery.

Bronchial segments (deflated at atmospheric pressure) were on average 2 mm and 3.5 mm in internal diameter at the distal and proximal ends respectively. Segments were cannulated at each end and then placed horizontally in an organ bath filled with gassed (5% CO₂ in O₂) Krebs solution (mM: NaCl 121; KCl 5.4; MgSO₄ 1.2; NaHCO₃ 25; sodium morpholinopropane sulphonic acid 5.0; glucose 11.5; and CaCl₂ 2.5) at 37°C (pH = 7.3). The airway segment was stretched and maintained at ~120% of its dissected length to approximate the length at functional residual capacity (Roca et al., 1998) and were on average ~16 mm after mounting (Figure 2.3). Details of bronchial segments are shown in Table 2.1.
Figure 2.3: Example of a human bronchial segment. The bronchial segment was cannulated at each end and mounted in an organ bath system filled with Krebs solution.

The lumen of the bronchial segment was mounted, horizontally, in a modified organ bath (custom made) connected to a jacketed reservoir containing Krebs solution to set the initial intraluminal pressure and therefore transmural pressure ($P_{tm}$) at $5\,\text{cmH}_2\text{O}$ (Figure 2.4). The opposite end of the segment was connected to a $1\,\text{mL}$ glass syringe (Model 1001, Hamilton, NV) filled with Krebs solution. Three-way taps at either end of the segment allowed flushing of the lumen as required. During measurements (see below) taps were closed to facilitate pressure / volume recordings and the simulation of breathing manoeuvers.
Table 2.1: Origin and dimensions of bronchial segments.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Lobe</th>
<th>Ls</th>
<th>V₀</th>
<th>Predicted diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary carcinoid tumour</td>
<td>RUL</td>
<td>9.5</td>
<td>145.2</td>
<td>5.0</td>
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<tr>
<td>Primary carcinoid tumour</td>
<td>RML+/-RLL</td>
<td>18.8</td>
<td>783.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Primary carcinoid tumour</td>
<td>RLL</td>
<td>4.8</td>
<td>59.7</td>
<td>5.2</td>
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<tr>
<td>Primary squamous cell carcinoma</td>
<td>RUL+/-RML</td>
<td>18.2</td>
<td>15.7</td>
<td>3.4</td>
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<tr>
<td>Left upper lobe of lung adenocarcinoma</td>
<td>LUL</td>
<td>13.6</td>
<td>92.0</td>
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<td>Secondary Metastatic osteosarcoma</td>
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<td>47.0</td>
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<td>Primary well differentiated adenocarcinoma</td>
<td>LUL</td>
<td>15.4</td>
<td>58.5</td>
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<td>Primary, keratinising squamous cell carcinoma</td>
<td>LUL</td>
<td>7.6</td>
<td>39.6</td>
<td>3.1</td>
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<td>Right Pneumonecotomy</td>
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<td>139.6</td>
<td>3.6</td>
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<td>Bronchial atresia</td>
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<td>20.1</td>
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<td>22.6</td>
<td>178.3</td>
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</tr>
<tr>
<td>Mycetoma</td>
<td>RUL</td>
<td>27.0</td>
<td>358.0</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**COPD**

| Squamous cell carcinoma                        | LUL           | 21.2| 44.1 | 4.1               |
| Primary poorly differentiated sarcomatoid carcinoma | LUL           | 23.6| 408.2| 5.3               |
| Metastatic single adenocarcinoma of colorectal origin | RLL           | 25.9| 486.0| 6.6               |
| Primary squamous cell carcinoma                | LUL           | 17.0| 105.8| 3.9               |
| Squamous cell carcinoma                        | RLL           | 18.0| 165.2| 4.4               |
| No information                                | LUL           | 14.3| 37.4 | 3.0               |

LUL = left upper lobectomy; RUL = right upper lobectomy; LLL = left lower lobectomy; RLL = right lower lobectomy; RML = right middle lobectomy; Ls = stretched length; V₀ = the volume of the fully relaxed airway at 0 cmH₂O determined in the organ bath (see experimental protocol); predicted diameter was calculated from V₀ and Ls approximating the airway as a uniform cylinder.

Pressure-volume recordings and simulation of breathing manoeuvres

A custom-built servo-controlled syringe pump and pressure transducer (Noble et al., 2007) was used to measure airway pressure and volume and to simulate breathing manoeuvers. Volume oscillation was achieved through movement of a syringe plunger, which was driven by a feedback controlled
servo motor (M540, Mclennan Servo Supplies, Surrey, UK) and motor controller (Shane De Catania, Perth, Australia) as shown in Figure 2.4. Airway P_{tm} was set and measured via a calibrated pressure transducer (model MLT0380/D; ADInstruments, Bella Vista, Australia). Changes in luminal volume were measured with a displacement transducer (HEDS-5540#A06, RS Components, Smithfield, Australia). Pressure and volume displacement were recorded by a PowerLab data acquisition system (model 4/30, ADInstruments) and displayed on a personal computer.

![Schematic representation of the custom-made organ bath system](image)

**Figure 2.4:** Schematic representation of the custom-made organ bath system. Transmural pressure was measured by a pressure transducer (P). Volume was measured through the movement of the syringe plunger and was driven by pressure feedback to the motor controller, allowing airway pressure to be set to desired static or oscillatory conditions.

**Experimental protocol**

Once in the organ bath, airways underwent assessment for leaks by gradually inflating the airway with a dye to ~50 cmH₂O followed by a 1 h equilibration period under P_{tm} of 5 cmH₂O. Tissue viability was confirmed via electrical field stimulation (30 Hz / 60 V / 3 ms) and acetylcholine (ACh, 10^{-4} M). Airways were allowed an additional 30 min of equilibration following viability assessment.

The two primary outcomes of the study were airway narrowing capacity to ACh and bronchodilation to DI. Cumulative dose-response curves to ACh (3 × 10^{-6} to 3 × 10^{-3} M) were constructed under static conditions (P_{tm} fixed at 5 cmH₂O) or dynamic conditions (simulating tidal breaths with intermittent DIs, see below) with the order of experiments randomized. Tidal breathing consisted of sinusoidal P_{tm} oscillations (5 to 10 cmH₂O, 0.25 Hz), and DI as a 2 s linear ramp up from 5 to 30 cmH₂O. This
was held at peak pressure for 2 s followed by another 2 s linear ramp down to 5 cmH\textsubscript{2}O (LaPrad \textit{et al.}, 2008, Noble \textit{et al.}, 2011).

The airways were pre-conditioned under dynamic conditions (~12 min), before dose-response curves to ACh were conducted to establish / set mechanical history. The pre-conditioning included tidal oscillation with DIs every 6 min, stimulating the rate of spontaneous breaths in human subjects (Bendixen \textit{et al.}, 1964). Bronchodilation to DI was assessed before and after airway narrowing to ACh. Bronchial segments were subsequently dilated maximally to theophylline (10\textsuperscript{-2} M) and the volume of the airway measured by withdrawal of the syringe until collapse, defined as the volume of the airway at 5 cmH\textsubscript{2}O (V\textsubscript{5}). The volume of the airway lumen at 0 cmH\textsubscript{2}O (V\textsubscript{0}) was determined from V\textsubscript{5} minus the volume required to deflate the airway to 0 cmH\textsubscript{2}O. The pressure (cmH\textsubscript{2}O) required to collapse the airways was also recorded. Airways were considered collapsed when a volume change at the distal end of the segment produced no change in pressure at the proximal end, showing the lumen was occluded between the ends of the segment (McFawn and Mitchell, 1997a).

\textit{Airway Morphometry}

Airways were fixed in 4\% formaldehyde following \textit{in vitro} experiments and the middle, distal and proximal end of the segments processed into wax blocks within a week after each experiment. From each block, transverse 4 μm sections were cut and stained with haematoxylin and eosin. Airway wall dimensions were then measured on the stained sections using morphometric software (newCAST version 4.2.1, Visiopharm A/S, Horsholm, Denmark) and included the perimeter of the basement membrane (P\textsubscript{bm}) and areas of the ASM, inner wall (W\textsubscript{ai}), outer wall (W\textsubscript{ao}) and total wall (W\textsubscript{at}).

To measure the volume fractions of ASM (V\textsubscript{VASM}), ECM (V\textsubscript{ECM}) and other components i.e., spaces due to fixation artefact, inflammatory cells and blood vessels (V\textsubscript{OTHER}), 0.5 μm sections were also cut from the wax blocks and stained with Masson’s trichrome stain (Jones \textit{et al.}, 2014). Under \texttimes 1,000
magnification, a grid was placed over the ASM layer, with each intersection representing a ‘point’ (Figure 2.5). Points were allocated to ASM (red), ECM (blue) and other (white) by their stain colour. Using the grid, the ASM layer was systematically and randomly sampled around each transverse section until 200 points were counted. The use of extremely thin sections prevented overlap from tissue appearing in different depth planes allowing a more precise estimate of muscle components.

![Figure 2.5: A magnified (× 1000) section of the airway smooth muscle (ASM) layer with the point counting grid overlayed. A 0.5µm section was stained with Masson’s trichrome stain and the point counting technique used to count the ASM (red), extracellular matrix (ECM; blue) and the other components (“Other”; white) within the ASM layer.](image)

**Analysis and statistics**

Changes in airway volume due to ACh were normalized to the fully relaxed airway volume measured after theophylline. Airway narrowing was expressed as the percentage decrease in luminal volume from the volume at 5 cmH2O transmural pressure (Ptm) – (%V5). Maximal response (E_max) was determined as the level of airway narrowing obtained at the highest ACh dose, which was $3 \times 10^{-3}$ M. Airway sensitivity to ACh (pD2; the negative logarithm of the concentration producing half-maximal response) was determined by fitting a sigmoidal dose-response curve to data generated from each individual bronchial segment. Bronchodilatory response to DI was dependent upon the specific protocol. Prior to airway narrowing induced to ACh, bronchodilation was calculated at baseline from
the increase in airway volume produced by DI (Post-DI – Pre-DI volume) divided by $V_S$. After induced airway narrowing to ACh, bronchodilation to DI was calculated as the percentage reversal of airway narrowing ($\Delta$ volume-DI / $\Delta$ volume-ACh) (Noble et al., 2011) where 100% reversal indicates complete abolishment of the ACh-induced airway narrowing. Airway compliance was also measured from tidal volume strains ($\Delta V / V_S$) divided by $\Delta P_{tm}$ (i.e., cmH$_2$O$^{-1}$).

Comparisons were between subjects with and without COPD. Two-way ANOVA was used to analyze airway narrowing and bronchodilation to DI (% reversal) across different ACh doses (repeat measures). Data were transformed where necessary to ensure that the assumptions of normality and homoscedasticity of variances were appropriate. Unpaired $t$-tests (with Welch’s correction as appropriate) were used to compare $pD_2$, baseline response to DI in vitro ($%V_S$) and in vivo response to DI (M/P ratio). Correlation between variables was determined using Pearson’s correlation coefficient. Graphpad Prism (version 7.02; Graphpad Software) and SigmaPlot (version 13.0; Systat Software) were used for graphical and statistical analyses. Data are mean ± standard error of the mean (SEM) with $p < 0.05$ as the level for statistical significance.
2.3 Results

Subject characteristics

Subject characteristics and lung function data are shown in Table 2.2. Our study group comprised of 13 males and 5 females, with 5 out of the 6 subjects classified as having COPD i.e., post-bronchodilator FEV₁ < 80% of the predicted value and an FEV₁/FVC ratio < 0.7, being males. Subjects were on average ~64 years of age, with no difference found in the mean age between the groups (p = 0.23).

All of the subjects exhibited some form of respiratory symptoms which included breathlessness, cough, phlegm, runny nose, wheeze and chest tightness. Previous respiratory illnesses included pneumonia (6), bronchitis (5), sinusitis (7) and hayfever (4). Six subjects were not using any medication and none of the subjects were on inhaled corticosteroids or bronchodilators. The remainder of the subjects were using a variety of cardiovascular medications. A history of smoking was observed in thirteen of our subjects with an average pack-years of 24 ± 11 in the control group and 46 ± 13 in the COPD group, which was not statistically different between the groups (p = 0.21).
Table 2.2: Subject characteristics and lung function parameters.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Smoking History</th>
<th>Pre-bronchodilator</th>
<th>Post-bronchodilator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FEV₁ %pred</td>
<td>FVC %pred</td>
</tr>
<tr>
<td>M</td>
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<td>Ex</td>
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<td>Current</td>
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<td>64 ± 8</td>
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<tr>
<td>M</td>
<td>73</td>
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<td>Ex</td>
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<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>69 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Ex = ex-smoker; Non = non-smoker; %pred = percent predicted. Bold text indicates average data including mean ± SEM where applicable.

Response to DI in vivo

The M/P ratio could not be determined in 2 control subjects due to difficulty in performing the manoeuvre. Subject M/P ratio was not altered by inhaled bronchodilator (p = 0.76). The M/P ratio (Figure 2.6A) was lower in the COPD group (0.45 ± 0.07) compared with the control group (0.87 ± 0.12; p = 0.02). All subjects diagnosed with COPD had an M/P ratio < 1, indicating that their response to DI was predominantly bronchoconstriction. For control subjects, 7 of the 10 subjects had an M/P ratio < 1. When all subjects were combined (Figure 2.6B), M/P ratio was positively correlated with FEV₁/FVC %predicted (r = 0.72, p = 0.002, n = 16), however no correlation was observed with FEV₁ %predicted and M/P ratio (r = 0.41, p = 0.12, n = 16).
Figure 2.6: Effect of deep inspiration in vivo. (A) maximal / partial (M/P) flow ratio in control (n = 10) and subjects with COPD (n = 6). M/P ratio was lower in the COPD group (p = 0.02); (B) M/P ratio was positively correlated with post-bronchodilator FEV₁/FVC (r = 0.72, p = 0.002; n = 16).
*Significantly different from control (p < 0.05). Data are mean ± SEM.

Airway narrowing and compliance

Under static conditions, increased narrowing of bronchial segments was observed in the COPD group (n = 6) compared with the control group (n = 12; Figure 2.7A). Maximal airway narrowing was 31.9 ± 3.8% of baseline volume in the control group and 51.2 ± 10.7% in the COPD group (p = 0.01). There was no difference in pD₂ between the control (3.9 ± 0.1) and COPD groups (4.1 ± 0.3; p = 0.52; Figure 2.7B). During simulated breathing (tidal oscillations prior to DI), Eₘₐₓ of bronchi from COPD patients (43.3 ± 8.7%) was still higher than control patients (24.6 ± 3.7%; p = 0.01) and again there was no difference in pD₂ between the control (3.7 ± 0.12) and COPD groups (3.9 ± 0.24; p = 0.31).

Bronchodilation (pharmacological) to theophylline was used as an indication of intrinsic ASM tone (i.e., contraction of the ASM in the absence of applied agonist) and was observed in 9 of the 12 airways from the control group (10.38 ± 2.8% increase in volume) and half of the airways in the
COPD group (10.1 ± 5.8% increase in volume). The magnitude of intrinsic tone between the groups was comparable (p = 0.96).

**Figure 2.7:** Airway narrowing and sensitivity (pD2) of bronchial segments under static conditions.

A) airway narrowing (change (Δ) in volume, %) dose-response curves to acetylcholine (ACh) performed in bronchial segments (control n = 12; COPD n = 6) under static conditions. Airway narrowing was increased at the three highest doses (p = 0.01); B) airway sensitivity (pD2, negative logarithm of the dose producing half maximal response) was comparable between the control and COPD groups (p = 0.52). *Significantly different from control (p < 0.05). Data are mean ± SEM.

Airway compliance was assessed from volume and pressure changes during tidal oscillation. Compliance in one subject with COPD was excluded due to system error. Baseline compliance (before ACh) was similar between control (0.018 ± 0.003 cmH2O⁻¹) and the COPD groups (0.019 ± 0.003 cmH2O⁻¹; p = 0.80). After contraction to a maximum dose of ACh, a reduction in compliance was observed in both groups (p < 0.0001; Figure 2.8). However, there was still no difference in compliance between groups (p = 0.92).
Figure 2.8: Compliance of bronchial segments. A reduction in the compliance of airways in both groups (control \( n = 12 \); COPD \( n = 5 \)) was observed (\( p < 0.0001 \)), however, the compliance between the groups was comparable (\( p = 0.92 \)). Data are mean ± SEM.

Response of the bronchial segments to simulated DI

Since the in vivo response to DI was assessed in the absence of induced bronchoconstriction (i.e., without bronchial challenge), the appropriate in vitro comparison is the response to DI prior to ACh administration. In contrast to the global in vivo response to DI, where bronchoconstriction prevailed, the response of the airway segment in vitro to DI (prior to ACh) was bronchodilation in all subjects (Figure 2.9A). The magnitude of bronchodilation to simulated DI in vitro was comparable between subjects (\( p = 0.60 \)). However, of particular interest, bronchodilation to DI in vitro was positively correlated with M/P ratio in vivo (Figure 2.9B).
Figure 2.9: In vitro and in vivo response to deep inspiration (DI) in control and COPD subjects.

A) magnitude of bronchodilation to DI (change (Δ) in volume, %) prior to acetylcholine (ACh) in bronchial segments in vitro was not different between the groups (control n = 12; COPD n = 6; p = 0.60); B) a positive correlation was observed between bronchodilation to DI in bronchial segments in vitro and the maximal / partial (M/P) ratio in vivo (r = 0.54, p = 0.03; n = 16). Data are mean ± SEM.

Bronchodilation to DI was also measured in vitro following ACh challenge as the percent reversal of induced airway narrowing. These measurements were performed at the three highest doses of ACh where airway narrowing was observed in all airways. No difference was found in the magnitude of bronchodilation between the groups (control n = 12; COPD n = 6; p = 0.75), however, bronchodilation was reduced with increasing doses of ACh in both groups (p = 0.01; Figure 2.10).
Figure 2.10: Bronchodilation to deep inspiration (DI) in bronchial segments. Magnitude of bronchodilation (% reversal) at the three highest doses was comparable between the two groups (control n = 12; COPD n = 6; p = 0.75), however, bronchodilation decreased with increasing doses of acetylcholine (ACh; p = 0.01). Data are mean ± SEM.

Table 2.3: Airway wall thickness.

<table>
<thead>
<tr>
<th></th>
<th>control (n = 10)</th>
<th>COPD (n = 6)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sqrt{ASM/P_{bm}}$</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.90</td>
</tr>
<tr>
<td>$\sqrt{W_{ai}/P_{bm}}$</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.97</td>
</tr>
<tr>
<td>$\sqrt{W_{ao}/P_{bm}}$</td>
<td>0.29 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.66</td>
</tr>
<tr>
<td>$\sqrt{W_{at}/P_{bm}}$</td>
<td>0.31 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.73</td>
</tr>
</tbody>
</table>

$\sqrt{ASM/P_{bm}} = \text{square root of airway smooth muscle normalized to perimeter basement membrane (}P_{bm}\text{); }\sqrt{W_{ai}/P_{bm}} = \text{square root of inner wall area normalized to perimeter basement membrane; }\sqrt{W_{ao}/P_{bm}} = \text{square root of outer wall area normalized to perimeter basement membrane; }\sqrt{W_{at}/P_{bm}} = \text{square root of total wall area normalized to perimeter basement membrane. Data are mean ± SEM.}$

Airway structure and morphometry

Data from two control subjects could not be obtained as the tissue was destroyed during processing. Airway wall areas and area of ASM were similar between subject groups (Table 2.3). The $V_{VECM}$ within the ASM layer (Figure 2.11A) was increased in the COPD group (0.24 ± 0.01) compared with
the control group (0.18 ± 0.02; p = 0.02). Inverse relationships were observed between $V_{\text{VECM}}$ and FEV$_1$ %pred ($r = -0.54$, $p = 0.03$; Figure 2.11B), FEV$_1$/FVC %pred ($r = -0.58$, $p = 0.02$; Figure 2.11C) and *in vivo* M/P ratio ($r = -0.61$, $p = 0.01$; Figure 2.11D). There were no correlations between $V_{\text{VECM}}$ and airway narrowing ($r = -0.08$, $p = 0.76$), baseline compliance ($r = 0.11$, $p = 0.67$), collapse pressure ($r = 0.17$, $p = 0.52$) or bronchodilation to DI ($r = 0.08$, $p = 0.77$). There were no statistical differences between $V_{\text{VASM}}$ in control (0.62 ± 0.02) and COPD groups (0.57 ± 0.01; $p = 0.12$), or $V_{\text{VOTHER}}$ in control (0.20 ± 0.01) and COPD groups (0.19 ± 0.01; $p = 0.30$).

**Figure 2.11:** Volume fraction of ECM ($V_{\text{VECM}}$) within the airway smooth muscle (ASM) layer and relationship to lung function and maximal / partial (M/P) ratio. A) within the ASM layer, $V_{\text{VECM}}$ was increased in subjects with COPD ($n = 6$) compared with control ($n = 10$; $p = 0.02$); B) $V_{\text{VECM}}$ was negatively correlated with FEV$_1$ %pred ($r = -0.54$, $p = 0.03$) and; C) FEV$_1$/FVC %pred ($r = -0.58$, $p = 0.02$); D) $V_{\text{VECM}}$ was negatively correlated with M/P ratio ($r = -0.61$, $p = 0.01$).

*Significantly different from control ($p < 0.05$; $n = 16$ for all correlations). Data are mean ± SEM.
2.4 Discussion

The present study compared the *in vitro* mechanical responses of bronchial segments from subjects with and without fixed airflow limitation i.e., COPD. Results show that airway narrowing capacity of the airway isolated from subjects with COPD exhibits an exaggerated bronchoconstrictor response to ACh compared with bronchial segments from the control group. While the response to simulated DI *in vitro* was not different between groups, it was predictive of the global response to DI observed *in vivo*. The only structural difference between groups was $V_{VECM}$ within the ASM layer that was increased in the COPD group, and inversely related to lung function and *in vivo* response to DI. These findings support mechanical changes to the airway wall in subjects with COPD that are potentially mediated by compositional changes to the ASM layer contributing to the onset of irreversible airway narrowing.

The main restriction on studies examining the mechanical properties of human airway tissue *ex-vivo* is access to lung tissue. Other laboratories have made use of transplant lungs that were not allocated to suitable recipients (Ijpma *et al.*, 2015). Our current approach is to utilize tissue acquired after the surgical removal of pulmonary neoplasms (Noble *et al.*, 2011), which was used to demonstrate increased narrowing of bronchial segments from subjects with a history of asthma (Noble *et al.*, 2013). There is an assumption that tumour pathology does not impact measurement outcomes, which seems appropriate since airways are dissected away from the tumour site and are removed from macroscopically normal tissue. Tumour pathology varied between subjects, but there was no overt difference between the control and COPD subjects. Both control and COPD subjects had a history of smoking and were taking medication for respiratory and non-respiratory ailments. Due to sample size, it is not possible to determine the effect of patient medication on experimental outcomes, nor can we rule out an effect from anaesthetics administered during surgery.
While recruitment from a surgical population allows the collection of some *in vivo* data prior to surgery, there are limits on the functional measurements that can be imposed on these subjects pre-operatively. Bronchodilatory response to DI is typically assessed after bronchoconstrictor challenge from the reversal of induced airway narrowing (Brown *et al.*, 2001). It was not possible, at least in this study, to perform bronchial challenge on subjects in the days preceding surgery. Response to DI was therefore assessed in the absence of induced tone using the M/P ratio, a variation of normal spirometry. It would have been preferable to relate bronchoconstrictor response and subsequent bronchodilation to DI *in vivo*, to comparable measures *in vitro*. Although, as will be discussed, the study has revealed some intriguing associations between M/P ratio *in vivo* and bronchodilatory response to simulated DI without induced tone *in vitro*. The surgical population also puts a limit on the severity of airflow limitation that our subjects are likely to exhibit. Surgeons on physical examination are less likely to operate on patients with severe airflow limitation and for this reason our subjects on average have mild to moderate, rather than severe COPD.

All airways studied were cartilaginous and therefore large conducting sized bronchi. A widely espoused viewpoint is that COPD is simply a disease of the small airways. Certainly, there is little doubt that small airway disease is a major contributor to airflow obstruction in some patients and includes loss and narrowing of these airways (Hogg *et al.*, 2009). Conversely, large airways are important in other COPD subjects. Patel *et al.* showed that airway wall thickness on computed tomography scan of airways with a lumen perimeter of 10 and 20 mm correlated independently (of emphysema score) with airflow obstruction in 3,096 patients (Patel *et al.*, 2008). Similarly, histological assessment of cartilaginous airways from 72 subjects showed an inverse relationship between inner wall area and FEV₁/FVC (Tiddens *et al.*, 1995). Finally, increased V_{VECM} in the ASM layer is expressed both in the small and large airways (Jones *et al.*, 2016a, Jones *et al.*, 2016b), suggesting a global disease process. These observations argue against the premise that COPD is purely a small airway disease.
A major finding of the present study is that bronchial segments from the COPD group exhibited increased maximal airway narrowing to ACh compared with the control group. Increased airway narrowing is consistent with the *in vivo* phenomenon of AHR which is of great importance in asthma (O’Byrne, 1986) and also documented in COPD (Postma and Kerstjens, 1998, Barnes, 2006). Unlike asthma, AHR in COPD (determined from the bronchoconstrictor dose producing a 20% fall in FEV$_1$) is related to a fall in baseline FEV$_1$/FVC (Ramsdale *et al.*, 1984, Yan *et al.*, 1985), suggesting that the exaggerated narrowing response is a pure geometric effect relating to reduced airway lumen calibre. Our findings instead support an intrinsic increase in the contractile responsiveness of the airway that may at least contribute to an exaggerated bronchoconstrictor response. *In vitro* studies on peripheral airways from human subjects with COPD show complimentary changes in ASM contractility to those presented in the current study. Opazo Saez *et al.* demonstrated an inverse correlation between ASM stress (force per cross-sectional area) and subject FEV$_1$/FVC (Opazo Saez *et al.*, 2000). Increased contractile response of both small and large airways may therefore contribute to AHR observed in COPD subjects *in vivo*.

Our group has now demonstrated increased airway narrowing capacity of large bronchial segments from subjects with asthma (Noble *et al.*, 2013) and COPD. In bronchial segments from asthmatic subjects, there was an increase in the thickness of the ASM layer and this was positively correlated with maximal airway narrowing. Increased airway narrowing in the asthma group was therefore attributed to greater force production and shortening due to the greater muscle bulk, as predicted in mathematical models (Lambert *et al.*, 1993, Affonce and Lutchen, 2006). In contrast, the thickness of the ASM layer in bronchial segments was not different between subjects with and without COPD, supporting an intrinsic change in ASM phenotype. The aforementioned study by Opazo Saez *et al.* (Opazo Saez *et al.*, 2000) provides direct supporting evidence for a change in ASM phenotype, since ASM stress production by definition reflects force normalized to muscle cross sectional area. Importantly, the current study shows that the same airways from COPD subjects which exhibited an
increased bronchoconstrictor response also had an increase in $V_{VECM}$ within the ASM layer, raising the possibility that changes in muscle composition are somehow related to contractile performance. Given that an increase in $V_{VECM}$ was detected, it is likely that there was some decrease in $V_{VASM}$ that was too small to detect in the current study. It is perhaps somewhat counterintuitive that a loss (albeit small) of contractile elements (ASM cells) at the expense of increased ECM could increase force production. The presence of ECM is however essential for the distribution of tension throughout the layer via cell-matrix coupling (Zhang and Gunst, 2008). Further studies are required to test the hypothesis that increased ECM composition modulates ASM force production and/or contributes to AHR.

Bronchodilation to DI (reversal of induced tone) is reduced in subjects with COPD (Scichilone et al., 2008, Scichilone et al., 2004) and has been associated with patient dyspnea (Wedzicha et al., 2012). Previous studies have demonstrated that emphysema contributes to the loss of bronchodilation to DI (Scichilone et al., 2005). The alternative explanation is that the response of the airway wall to mechanical stretch is altered in COPD. Bronchodilatory effects of DI are largely attributed to reduced ASM force after stretch (Noble et al., 2012, Shen et al., 1997) due to cross-bridge detachment (Fredberg et al., 1997, Fredberg et al., 1999) or depolymerisation of contractile filaments (An et al., 2002). In subjects with asthma, there is mounting evidence that the ASM responds differently to stretch (Noble, 2013, Brown et al., 2001, Chin et al., 2012, Hulme et al., 2013). We compared bronchodilatory response to simulated DI in bronchial segments from subjects with and without COPD, both before and after induction of exogenous tone with ACh. A DI was defined as per previous studies (Noble et al., 2011, Noble et al., 2013) as an increase in $P_{tm}$ from 5 to 30 cmH$_2$O, the plateau of the lung pressure-volume curve, a rough approximation of what the airway may experience during maximal inflation in vivo. Prior to ACh, bronchodilation to DI was observed, which reflects passive hysteresis or reversal of intrinsic ASM tone. Intrinsic tone (~10%) was detected in the present study and was not noticeably different between groups. The bronchodilatory response after DI at baseline
was also not different between groups. Similar findings were observed after the induction of ASM tone to ACh, where the percent reversal of airway narrowing was not different between groups, despite greater airway narrowing in the COPD group. There is therefore no evidence that the response of the airway wall to stretch differs between subjects with and without COPD.

Putting aside a potential phenotypic difference in the ASM response to stretch, reduced airway compliance (increased stiffness) could also reduce the response of the airway wall to DI. Increased airway stiffness as a result of remodelling or increased ASM tone has been proposed in asthma (Ward et al., 2001, Baldi et al., 2010) and there is considerable evidence showing that airway wall stiffening reduces bronchodilation to dynamic breathing manoeuvres (Harvey et al., 2015, Noble et al., 2011). Airway wall stiffening has also been identified in subjects with COPD, though less frequently and dependent on disease severity. High resolution computed tomography was used to show a decrease in the distensibility (inversely related to airway stiffness) of large to medium sized airways in subjects with GOLD stage II and IV COPD (Scichilone et al., 2008). Measurements of airway compliance in the present study were not different between groups and does not support a change in airway stiffness in COPD. While this is consistent with the comparable response to DI between groups, our measurements were derived from small tidal-equivalent pressure fluctuations (Δ 5 cmH2O). Experimentally induced loss of collagen is known to increase airway expansion to large inflating pressures without changing the compliance at moderate pressures (Karlinsky et al., 1976). Hence, pressure-volume assessment across a broader range may have provided more comprehensive information on changes in airway compliance / stiffness.

As discussed, bronchial challenge (in vivo) was not performed, which prevented any comparisons with in vitro responsiveness or the assessment of the magnitude to which DI could reverse bronchoconstriction. Instead, response to DI was assessed from M/P ratio without bronchial challenge. In control subjects, M/P ratio was close to 1, indicating that there was little global
bronchoconstriction nor bronchodilation to DI. This is consistent with other studies which show that in the absence of bronchial challenge, M/P ratio supports no real change in airflow (Pellegrino et al., 1998, Brusasco et al., 1992). Some bronchodilation still likely occurs at the airway level due to passive hysteresis or reversal of intrinsic tone, but is offset by parenchymal hysteresis that favours a reduced lung elastic recoil pressure (and therefore constriction). The balance between airway and parenchymal response to stretch is referred to as the airway-parenchymal hysteresis theory (Froeb and Mead, 1968). Our results are in fact a nice demonstration of the airway-parenchymal hysteresis theory since in vitro dilation to DI (%V5 before ACh administration) was positively related to M/P ratio. This observation is intuitive since greater bronchodilation (or airway hysteresis) should increase M/P ratio even if parenchymal hysteresis still dominates, such that M/P ratio does not exceed 1.

Subjects with COPD exhibited a lower M/P ratio compared with the control group, supporting a greater global bronchoconstrictor response. These differences do not appear to be explained by a change in the airway wall response to stretch, as this was not altered in the in vitro studies. On the other hand, increased VVECM predicted a lower M/P ratio in vivo but did not affect in vitro airway response. These results are consistent with a change in airway-parenchymal interactions in subjects with COPD. Increased VVECM within the ASM layer may not necessarily regulate the in vivo response to DI but could be co-related with other pathologies, in particular emphysema which was not assessed in these subjects. Airway anisotropy could also be a factor since in vitro experiments apply a positive pressure from the inside of the airway rather than a negative pressure from the outside of the airway. If airway tissue responds differently to external versus internally applied pressure, which to our knowledge has never been directly tested (due to methodological limitations), then VVECM within ASM layer may show associations with in vivo but not in vitro response, through a mechanism relating to the altered distribution of externally applied ASM tension.
In summary, the major finding from the present study is that large bronchial segments from subjects with COPD exhibit an exaggerated airway narrowing response consistent with AHR. The underlying pathology is increased $V_{\text{VECM}}$ within the ASM layer without any change in gross wall thickness. Changes in ECM composition were directly related to in vivo response to DI and we propose may contribute to differences in ASM force production (and in turn AHR) which should be tested in future investigations. Finally, these data reinforce the need to consider the large airways in disease pathogenesis and in the development of targeted therapies.
Chapter 3: Structural composition of the smooth muscle layer determines the magnitude of intrinsic tone in human peripheral airways.

3.1 Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by airflow limitation that is not reversible by conventional therapies. The development of airflow limitation is mediated by the narrowing and loss of small airways (McDonough et al., 2011), narrowing of large airways (Kurashima et al., 2013) and emphysema (Hogg et al., 2009). Narrowing of the airway lumen is partly geometrically mediated as a result of increased wall thickness (Patel et al., 2008, Tiddens et al., 1995), including expansion of the airway smooth muscle (ASM) layer (Kuwano et al., 1993). Excessive contraction of the ASM (i.e., airway hyper-responsiveness, AHR) may also contribute to airflow limitation in COPD (O'Byrne, 1986), although whether there are real differences in airway narrowing capacity in COPD is a subject of debate, since bronchoconstrictor response is strongly dependent on resting lung function (Ramsdale et al., 1984, Yan et al., 1985). However, we have recently demonstrated an increase in bronchoconstrictor response in bronchial segments from subjects with COPD (Chapter 2), suggesting that contractile properties of the ASM layer do contribute to airflow limitation in COPD.

Changes in the mechanical behaviour or contractile capacity of the ASM in airway disease has been previously considered, and is frequently debated in the context of asthma (Bosse et al., 2012, Gunst and Panettieri, 2012). While the number of studies examining ASM contraction in COPD are far less than in asthma, there is evidence that the mechanical phenotype of ASM is different in subjects with COPD. Opazo Saez et al. studied peripheral bronchial rings and demonstrated a negative association between ASM stress (force per ASM area) in response to acetylcholine (ACh) with baseline lung function (Opazo Saez et al., 2000). Such observations are consistent with the possibility that increased contractility of the ASM contributes to AHR. However, common clinical observations are not
necessarily consistent with the notion of increased ASM contractility, in that the vast majority of subjects with COPD do not exhibit a response to inhaled bronchodilator (Wedzicha et al., 2013), which may indicate that the ASM exists in a low contractile state.

Changes in ASM contraction in COPD could be mediated by a shift in the structural composition of the ASM layer. The proportion of extracellular matrix proteins (ECM) within the ASM layer is increased in subjects with COPD and is inversely related with baseline lung function (Jones et al., 2016b) (Chapter 2). The change to the ASM layer in subjects with COPD is in contrast with asthma, where there is no change in muscle composition (James et al., 2012), despite a significant increase in muscle mass. The proportion of ECM within the ASM layer could modify ASM mechanics by affecting the transmission of force within the ASM layer and to other tissues (Zhang and Gunst, 2008). Alternatively, as ECM increases, there may be proportionally less contractile elements within the ASM layer which should influence the normal contractile state of the ASM i.e., intrinsic ASM tone, in the absence of an exogenous stimulus. The relationship between the composition and mechanical behaviour of the ASM layer has not been examined previously and is therefore the focus of the present study.

In order to acquire human airway tissue for ex-vivo study, we recruited subjects undergoing lung resection surgery and obtained baseline lung function from patient charts. Peripheral airways obtained following surgery were studied in organ bath chambers to assess ASM contraction to ACh and histamine (His), and relaxation to theophylline, the latter of which established the presence of intrinsic tone. Mechanical properties of the ASM were correlated against volume fractions of ASM ($V_{VASM}$) and ECM ($V_{VECM}$) within the ASM layer. We hypothesized that muscle composition would predict the magnitude of induced and intrinsic ASM tone.
3.2 Methods

Subject characteristics and lung function

Subjects (n = 90) undergoing lung resection surgery to remove tumours were recruited on the day of surgery from 4 hospitals in Perth, Western Australia, Australia. Pre-bronchodilator lung function (and in some subjects, post-bronchodilator) was performed 2-3 weeks prior to surgery. Subjects were classified as having airflow limitation if they had a post-bronchodilator (3 puffs of salbutamol from a metered dose inhaler, 100 μg/puff) FEV₁/FVC ratio < 0.7 and FEV₁ < 80% predicted. Reference equations for predicted values were from the 3rd National Health and Nutrition Examination Survey (Hankinson et al., 1999). Subjects completed a respiratory health questionnaire including general information about any illnesses, medications used and smoking history. All subjects provided informed consent to participate in the study and approval for the study was obtained from the Human Research Ethics Committees of Sir Charles Gairdner Hospital, Mount Hospital, Hollywood Hospital and Fiona Stanley Hospital (Approval number 2013-227).

Airway preparation

From the 90 subjects recruited for the study, suitable tissue for in vitro examination was obtained from 18 subjects. Tissue obtained post-operatively was macroscopically normal and acquired away from the affected area of the lung. Peripheral airway rings (1-4 per subject based on availability) were dissected from the resected tissue and mounted in organ bath chambers of a DMT myograph system (620M) (Figure 3.1). Each bath was fitted with two mounting pins: one connected to a micrometer that allowed the perimeter of the ring to be changed; the second to a transducer that recorded isometric force. Force signals were exported through a PowerLab data acquisition system (ADInstruments). Organ bath chambers were filled with gassed (5% CO₂: 95% O₂) Krebs solution (mM: NaCl 121; KCl 1.4; MgSO₄ 1.2; NaHCO₃ 25; sodium morpholinopropane sulphonic acid 5.0; glucose 11.5; and CaCl₂ 2.5) at 37ºC (pH = 7.3).
**Figure 3.1: Schematic of the organ bath system.** Airway rings were mounted in the organ bath filled with gassed and heated Krebs solution. Rings were drawn over a pair of stainless steel pins, where one pin was connected to a micrometer and the other to a force transducer. All rings were initially stretched to a tension of 0.1 g and the linear distance between the external surface of the pins was defined as $L_{ref}$ (reference length). Width ($w$) of the ring, required to calculate tension and stress, is indicated.

Full details of airway rings are shown in Table 3.1. Initial diameters of the unstressed rings were determined from the micrometer length (in mm) at which airway tension (see experimental protocol below) was first identified. Absolute micrometer length was calibrated to a graticule under a dissecting microscope. The dissecting microscope was also used to measure the width of the airway rings at the end of the experiment which was used to calculate ASM cross-sectional area and subsequently stress (see Analysis and statistics). The majority of rings contained only small amounts of cartilage, with some none at all (i.e., they were peripheral bronchioles).
Table 3.1: Origin and dimensions of airway rings.

<table>
<thead>
<tr>
<th>Lobe</th>
<th>Number of rings</th>
<th>Width mean (range) mm</th>
<th>Diameter mean (range) mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUL</td>
<td>4</td>
<td>2.5 (2.1-2.9)</td>
<td>1.1 (1.0-1.2)</td>
</tr>
<tr>
<td>RUL</td>
<td>4</td>
<td>2.3 (1.9-3.1)</td>
<td>0.9 (0.8-1.0)</td>
</tr>
<tr>
<td>RLL</td>
<td>2</td>
<td>2.0 (1.8-2.2)</td>
<td>0.6 (0.6-0.6)</td>
</tr>
<tr>
<td>LUL</td>
<td>1</td>
<td>1.1 (1.1)</td>
<td>0.9 (0.9)</td>
</tr>
<tr>
<td>LUL</td>
<td>2</td>
<td>2.2 (2.0-2.3)</td>
<td>0.7 (0.7)</td>
</tr>
<tr>
<td>LUL</td>
<td>2</td>
<td>1.8 (1.4-2.0)</td>
<td>0.8 (0.7-0.8)</td>
</tr>
<tr>
<td>RLL</td>
<td>1</td>
<td>1.6 (1.6)</td>
<td>0.6</td>
</tr>
<tr>
<td>LUL</td>
<td>1</td>
<td>1.9 (1.9)</td>
<td>0.6</td>
</tr>
<tr>
<td>RLL</td>
<td>3</td>
<td>1.9 (1.7-2.1)</td>
<td>0.8 (0.8-0.9)</td>
</tr>
<tr>
<td>RML</td>
<td>1</td>
<td>2.1 (2.1)</td>
<td>0.7</td>
</tr>
<tr>
<td>Right pneumonectomy</td>
<td>3</td>
<td>2.2 (2.0-2.5)</td>
<td>0.8 (0.6-1.0)</td>
</tr>
<tr>
<td>RUL</td>
<td>1</td>
<td>1.5 (1.5)</td>
<td>0.9</td>
</tr>
<tr>
<td>RLL</td>
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<td>2.1 (2.1)</td>
<td>0.8</td>
</tr>
<tr>
<td>RLL</td>
<td>3</td>
<td>1.7 (1.4-2.0)</td>
<td>0.7 (0.7-0.8)</td>
</tr>
<tr>
<td>LLL</td>
<td>3</td>
<td>2.0 (1.8-2.4)</td>
<td>0.6 (0.5-0.7)</td>
</tr>
<tr>
<td>LLL</td>
<td>2</td>
<td>2.0 (1.8-2.1)</td>
<td>0.6 (0.6)</td>
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<tr>
<td>LLL</td>
<td>1</td>
<td>1.6 (1.6)</td>
<td>0.5</td>
</tr>
<tr>
<td>RUL</td>
<td>1</td>
<td>2.3 (2.3)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

LUL = left upper lobectomy; RUL = right upper lobectomy; LLL = left lower lobectomy; RLL = right lower lobectomy; RML = right middle lobectomy. Width was determined at the end of the experiment under a microscope; Diameter was determined from the micrometer length at which tension was first detected. Data shown are case means and ranges (in parentheses).

Experimental protocol

After mounting in the organ bath, airway rings were gradually stretched to a pre-load of ~0.1 g over a 1 min period. The micrometer length was then determined and denoted as $L_{ref}$. Under tension, airway rings should essentially be considered as flattened tubes (Fig. 3.2) where diameter is equal to
perimeter divided by \( \pi \). For simplicity, the use of length in this study refers to lumen length determined by the micrometer.

**Figure 3.2:** *Image of a thin-walled airway ring (transverse view), acquired after fixation in the organ bath. The airway is in a stretched state following a length-tension protocol to identify relative optimum length. The white line indicates equivalent lumen length as determined by the micrometer. The bulbs at the ends of the line are representative of the perpendicular mounting pins.*

Airway rings were allowed to equilibrate at \( L_{eq} \) for \( \sim 1 \) h, before tissue viability was confirmed to ACh \( (10^{-4} \text{ M}) \). Length-tension curves to KCl \( (60 \text{ mM}) \) were constructed whereby length was increased in 20\% increments (NB. the rate of length change varied between tissues but was approximately 5 s for each increment), until a relative optimum length of contraction \( (L_o) \) was determined (Figure 3.3). The use of KCl was preferred to ACh as this reduced the risk of receptor desensitization to the applied agonist. Once \( L_o \) was confirmed by reduced force production with increasing length, the tissue was returned to \( L_o \). A period of ‘length-adaptation’ followed (Bosse et al., 2008, Wang et al., 2001), which comprised of repeated isometric contractions to KCl \( (60 \text{ mM}) \). Each ring was contracted 4 times, separated by 5 min rest periods. Contractions were slow to reach peak and equally slow to relax \( (~20 \text{ min per contraction / relaxation cycle}) \). The duration of the adaptation period was therefore \( \sim 2 \) h.

At the end of the adaptation period, cumulative dose-response curves to ACh \( (10^{-8} \text{ M} – 3 \times 10^{-3} \text{ M}) \) were constructed. The drug was then washed out and the airway allowed to spontaneously relax by
regularly changing the Krebs solution in the organ bath. From that point onwards, airways were exposed to Krebs solution containing atropine (10^{-6} M) to prevent neurological effects associated with subsequent exposure to His (Brink et al., 1980). After 20 min of exposure to atropine, a near-maximal dose of ACh (10^{-4} M) was administered to confirm cholinergic blockade. Dose-response curves to His (10^{-8} M - 3 \times 10^{-3} M) were then constructed, followed by drug removal and relaxation. Finally, total ASM relaxation including removal of intrinsic ASM tone (contractile state of the ASM layer in the absence of an exogenous stimulus) was produced by the addition of theophylline (10^{-2} M) for ~5 min. Airways were fixed in the organ bath (4% formaldehyde diluted in Krebs solution) for morphometry.

![Length-force relationship for a single airway ring](image)

**Figure 3.3:** Length-force relationship for a single airway ring. The raw passive force (g) is plotted as a function of lumen length (mm). Active isometric force was obtained by subtracting the passive tension from the total tension measured. Active force to KCl was measured until subsequent increases in length produced a decrease in force. Active data was predicted by a second order polynomial equation, and passive data by an exponential equation. Optimum length (L_o) was defined as that producing maximal active force, as indicated in the figure.

**Airway morphometry**

Fixed airways were processed and embedded into wax blocks within a week after each experiment. From each block, transverse airway sections of 0.5 μm and 4 μm were cut and stained with Masson’s
trichrome, and haemotoxylin and eosin stains respectively. Airway wall dimensions were then measured on the stained sections as previously described (Chapter 2), and included the perimeter of the basement membrane (P_{bm}) and area of the ASM layer. The V_{VECM}, V_{VASM} as well as “other” (V_{OTHER}) were measured on the 0.5 μm sections by point counting (Jones et al., 2014) (Chapter 2). “Other” includes space due to fixation artefact, inflammatory cells and blood vessels.

Analysis and statistics

Since force recordings in ring preparations are effectively double that present in an intact bronchial segment (i.e., both sides of the ring contribute to the measured force), recorded forces were halved. Force measurements were normalized to ring width (mN/mm, i.e., tension) or ASM cross-sectional area (i.e., stress in mN/mm²). Cross-sectional area (of the ASM layer) was determined from ring width multiplied by ASM thickness, measured histologically. Thickness of the ASM layer was defined as muscle area measured in the transverse plane divided by P_{bm} (thickness = area / P_{bm}; mm). Airways had a mean P_{bm} of 6.4 ± 0.4 mm (n = 18).

Sigmoidal dose-response curves were fit to stress data recorded after ACh and His, which allowed calculation of maximal stress (mN/mm²) and sensitivity (pD₂), the negative logarithm of the concentration producing half-maximal response. Intrinsic tone was determined from the change in tension / stress produced by theophylline, relative to the post-adaptation time-point, prior to the addition of either contractile agonist. Measurements were performed for each individual ring (1-4 rings per subject) and then expressed as case / subject mean. Differences in maximal stress and sensitivity between ACh and His were assessed using Student’s paired t-test. Correlations between morphological and physiological variables was determined using Pearson’s correlation coefficient or non-parametric Spearman’s correlation, depending on normality. Graphpad Prism (version 7.02; Graphpad Software) was used for data analysis and graphical representation. Data are mean ± standard error of the mean (SEM) with p < 0.05 as the level for statistical significance.
3.3 Results

Subject characteristics

Patient demographics as well as lung function data are shown in Table 3.2. On average, most of the subjects had relatively normal lung function relative to their age with FEV$_1$ %pred of 93 ± 4 and FEV$_1$/FVC of 0.73 ± 0.02. Two subjects had airflow limitation defined as an FEV$_1$ < 80% of the predicted value and an FEV$_1$/FVC ratio < 0.7. One of those subjects still had airflow limitation after bronchodilator, while the 2nd did not have post-bronchodilator lung function assessed. Most of the subjects had a history of smoking with an average pack-years of 21 ± 7. Six subjects had no prior respiratory symptoms while others reported instances of breathlessness, cough, phlegm, wheeze and chest tightness. Previous illnesses included pneumonia (1), bronchitis (4), sinusitis (3) and rhinitis (4). Nine subjects were not using any medication, and no subject was on inhaled corticosteroids or bronchodilators. The remainder of the subjects were using a variety of anti-depressant and cardiovascular medications.
Table 3.2: Subject characteristics.

<table>
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<tr>
<th>Age</th>
<th>Sex</th>
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<th>Post-bronchodilator</th>
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<td>Ex</td>
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<tr>
<td>61</td>
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<td>Non</td>
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<td></td>
<td></td>
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<td>72</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
<td>62</td>
</tr>
</tbody>
</table>

|M = male; F = female; %pred = percent predicted; non = Non-smoker; Ex = Ex-smoker.*Subject with fixed airflow limitation.

Active response to contractile agonists

Cumulative dose-response curves to ACh and His (10$^{-8}$ M – 3 × 10$^{-3}$ M) were performed (Figure 3.4A and 3.4B) for each ring and averaged i.e., to generate a case mean which was taken as the result for that subject. Peripheral airway rings (n = 18 subjects) exhibited an average maximal stress to ACh and His of 70.04 ± 9.73 and 76.34 ± 9.40 mN/mm$^2$ respectively (Figure 3.5A), which was not
statistically different (p = 0.11). There was also no difference in sensitivity (pD$_2$) between ACh (5.05 ± 0.19) and His (5.37 ± 0.22; p = 0.07; Figure 3.5B).

![Figure 3.4](image)

**Figure 3.4:** Example tension dose-response curves from an individual subject: (A) acetylcholine (ACh); and (B) histamine (His). Three rings were studied from this subject. Data from each ring were normalized to airway smooth muscle cross-sectional area (stress = mN/mm$^2$) and then averaged to generate a case mean.

![Figure 3.5](image)

**Figure 3.5:** Maximal stress and sensitivity (pD$_2$) of airway rings. No difference was found in A) maximal stress (p = 0.11); or B) sensitivity (p = 0.07) between acetylcholine (ACh) and histamine (His). Data are mean ± SEM (n=18).
Intrinsic tone

Changes in tension prior to the administration of a contractile agent (referred to as passive tension in the present study) reflects variable intrinsic ASM tone. Figure 3.6 shows the changes in passive tension throughout the experimental protocol. Confirmation of L_o required stretching the airway past the point of maximal force generation, which also allowed us to examine changes in passive tension after returning the airway to L_o. Passive tension decreased after return to L_o (p < 0.0001) compared with the initial tension when the airway was first stretched to L_o. Following return to L_o, the passive tension gradually re-developed during the adaptation period. The passive tension prior to the ACh and His dose-response curves were both comparable to that observed at L_o (p = 0.64). Due to the simultaneous increase in passive and total tension during the length-adaptation period (Figure 3.7A), there was no change in active tension (p = 0.15; Figure 3.7B).

Figure 3.6: Passive tension over the course of the experiment. Once airways were returned to relative optimum length for contraction (L_o), passive tension decreased and gradually re-developed during adaptation. Tension after relaxation to theophylline was significantly lower compared with prior acetylcholine (ACh) or histamine (His) (p < 0.0001). *p < 0.05 compared with L_o; #p < 0.05 compared with theophylline. Data are mean ± SEM (n=18).
To quantify intrinsic tone, airways were relaxed to a high dose of theophylline (10^{-2} M), producing a substantial decrease in passive tension, below that present prior to the ACh and His (p < 0.0001) dose-response curves. Intrinsic tone was calculated as the decrease in tension / stress produced by theophylline compared to that prior to the ACh dose-response curve. The endogenous source of intrinsic tone was not examined, although atropine transiently decreased intrinsic tone below the level present before administration of ACh (p = 0.0004).

**Figure 3.7: Changes in tensions during the adaptation period.** A) total tension (mN/mm) decreased from optimum length for contraction (L_o), but gradually increased throughout the adaptation protocol (p = 0.0005); B) no change in active tension (mN/mm) was observed throughout the adaptation period at L_o (p = 0.25). A1, A2, A3 and A4 refer to the first, second, third and fourth contraction to KCl once the airway was returned to L_o. *p < 0.05 compared with A1; #p < 0.001 compared with A1. Data are mean ± SEM (n = 18).

When normalized to muscle cross-sectional area, bronchial rings exhibited a mean intrinsic stress of 28.8 ± 5.1 mN/mm^2, which was about 30% of the total stress increase produced by maximal doses of ACh or His (Figure 3.8). Intrinsic stress was positively correlated with maximal response to ACh (r = 0.55, p = 0.02) but not His (r = 0.30, p = 0.22; Figure 3.9).
**Figure 3.8:** Intrinsic and induced airway smooth muscle (ASM) stress. Intrinsic stress was ~30% of the stress increase produced by acetylcholine (ACh; \( p = 0.0002 \)) or histamine (His; \( p < 0.0001 \)). Data are mean ± SEM (\( n = 18 \)).

**Figure 3.9:** Relationship between stress produced by acetylcholine (ACh) or histamine (His) and intrinsic stress (mN/mm\(^2\)). A) a positive correlation was observed between intrinsic stress and stress produced by ACh (\( r = 0.55, p = 0.02 \)); B) intrinsic stress and stress produced by His were not related (\( r = 0.30, p = 0.22 \); \( n = 18 \) for all correlations).
**Effect of ASM composition on contraction**

No correlations were found between stress produced by ACh and $V_{VASM}$ ($r = -0.04, p = 0.88$); $V_{VECM}$ ($r = 0.07, p = 0.79$); or $V_{VOTHER}$ ($r = 0.11, p = 0.67$). There was similarly no relationship between the composition of the ASM layer and stress produced to His: $V_{VASM}$ ($r = -0.21, p = 0.51$); $V_{VECM}$ ($r = 0.11, p = 0.67$); and $V_{VOTHER}$ ($r = 0.19, p = 0.44$). However, intrinsic stress was related to muscle composition (Figure 3.10). There was a positive correlation between $V_{VASM}$ and intrinsic stress ($r = 0.55, p = 0.02$; Figure 3.10A) and an inverse relationship observed between intrinsic stress and $V_{VECM}$ ($r = -0.52, p = 0.03$; Figure 3.10B). No correlation was found between intrinsic stress and $V_{VOTHER}$ ($r = -0.06, p = 0.81$). Total stress, calculated from intrinsic and agonist induced contraction tone (to either ACh or His), was not related at all to muscle composition.

![Graph A](image1.png)

**Figure 3.10: Effect of muscle composition on intrinsic stress.** A) a positive correlation was observed between the volume fraction of airway smooth muscle ($V_{VASM}$) and the magnitude of intrinsic stress (mN/mm$^2$; $r = 0.55, p = 0.02$); B) An inverse relationship was found between intrinsic stress and volume fraction of extracellular matrix ($V_{VECM}$) within the muscle layer ($r = -0.52, p = 0.03$; $n = 18$ for all correlations).

**Relationship between in vivo and in vitro function**

We used measurements of pre-bronchodilator FEV$_1$ %pred and FEV$_1$/FVC due to the greater sample size, as continuous variables ($n = 18$) to examine the relationship with ASM stress. No correlation
was found between average stress produced by ACh and FEV$_1$ %pred ($r = -0.03$, $p = 0.91$) or FEV$_1$/FVC ($r = 0.32$, $p = 0.20$). Similarly, no relationship was found between average stress produced by His and FEV$_1$ %pred ($r = 0.04$, $p = 0.88$) or FEV$_1$/FVC ($r = 0.41$, $p = 0.09$). The relationship between average intrinsic stress (mN/mm$^2$) and lung function parameters was also assessed. No significant correlations were found between intrinsic stress and FEV$_1$ %pred ($r = 0.19$, $p = 0.45$) and FEV$_1$/FVC ($r = 0.22$, $p = 0.38$). Correlations between lung function parameters and airway structure was also assessed, however, no relationship was found between $V_{VASM}$, $V_{VECM}$, $V_{OTHER}$ and FEV$_1$ %pred and FEV$_1$/FVC ($p > 0.05$).

### 3.4 Discussion

The purpose of this study was to determine whether the structural composition of the ASM layer alters the contractile behaviour of the airway wall. We measured the contractile response to ACh and His and the relaxant response to theophylline in human airway rings obtained from the lung periphery. No correlations were found between induced ASM stress and muscle composition. However, peripheral airways exhibited a substantial amount of intrinsic tone which positively correlated with $V_{VASM}$ and inversely with $V_{VECM}$ within the ASM layer. The magnitude of intrinsic stress was also correlated with subsequent contractile response to ACh. Together these findings support a relationship between composition of the ASM layer and the mechanical behaviour of the airway.

Peripheral airway rings were acquired from subjects undergoing resection surgery to remove tumours. Most subjects approached were happy to participate in the study (~85%), but lung tissue could only be obtained from 40 subjects due to tumour location or availability of a pathologist to provide tissue. Of the 40 lung samples obtained, 18 contained peripheral airways that were successfully dissected. The majority of subjects had relatively normal lung function. Only 2 subjects had airflow limitation, one of which had post-bronchodilator data available and could therefore be classified as having fixed airflow limitation (i.e., COPD). It was therefore not possible to assess the effect of COPD per se on
experimental outcomes, and it was not surprising that lung function was not related with in vitro ASM mechanics (Opazo Saez et al., 2000) or volume fractions within the ASM layer, as previously demonstrated (Jones et al., 2016b) (Chapter 2).

Unlike our previous studies where human airways were studied on the day of collection (Noble et al., 2011, Noble et al., 2013), the present study examined airways after overnight storage. The decision to store the tissue was not only for pragmatic reasons, but to allow a washout period in the event that anaesthetic agents administered during surgery could impact ASM function (Hulsmann and de Jongste, 1993). The limitation of this approach is that mechanical unloading (after dissection from the parenchyma) will have some influence on ASM contraction, likely mediated through ASM length-adaptation (Naghshin et al., 2003). For this reason, the experimental protocol included a careful preconditioning component where relative optimum length was first established followed by a period of length-adaptation to repeated KCl stimulations. While we cannot be certain that the contractile state of the ASM mirrors what would occur in vivo, the mechanical history was well standardized across all rings so that the effects of structure on ASM contraction could be determined.

The present study was motivated by our previous findings where narrowing capacity was increased in bronchial segments from subjects with COPD which also showed an increased V_{VECM} (Chapter 2). Increased contractility of the ASM could explain these observations (Opazo Saez et al., 2000) and for this reason we focused on examining the relationship between contractions induced under isometric conditions, rather than shortening, which is affected by after-loads from within (discussed below) and outside the ASM layer. Contractile responses were to ACh and His, which act directly on the ASM, although the latter also operates through a vagal reflex (Vidruk et al., 1977). To prevent activation of vagal nerve endings, atropine was administered and as such His responses reflected direct agonist actions on the ASM. The contractile response to ACh and His were comparable in terms of both maximal response and sensitivity. Comparisons with other studies are difficult due to
differences in methodologies. Without atropine pre-treatment, sensitivity to ACh and His in bronchial tissue were shown to be comparable (Brink et al., 1980), while in a different study maximal response was greater to ACh than His (Ghelani et al., 1980).

Maximal contractile stress induced to ACh and His is comparable with previous literature. Opazo Saez et al. reported a maximal ASM stress ~50 mN/mm² from non-obstructed subjects in peripheral airways, although this was to a 10⁻⁵ M ACh dose, which is likely to be below peak stress, despite the authors’ claims that this represents a supramaximal dose (Opazo Saez et al., 2000). We report a maximal response of ~75 mN/mm² with a pD₂ of ~5, which would equate to 35-40 mN/mm² at the same dose used by Opazo Saez et al. This is somewhat lower than in their study, but reasonably consistent allowing for differences in tissue collection, methodology and variable intrinsic tone. In other studies on ASM from more proximal airways, maximal stress is reported as ~80 mN/mm² (Chin et al., 2010) and ~160 mN/mm² (Chin et al., 2012) to electrical field stimulation, and ~120 mN/mm² to 10⁻⁴ M methacholine (Ijima et al., 2015). Together these data seem to suggest that the stress producing capacity of ASM from proximal airways is greater than more distally located airways, which is not consistent with recent data from horses where tracheal and peripheral ASM generated comparable stress (Matusovsky et al., 2016).

A primary aim of the study was to determine whether muscle composition, particular ECM affected contractile response. We ultimately propose that changes in the coupling state between cells and matrix within the muscle layer could impact the response to contractile agonists, and potentially explain increased ASM stress in peripheral airways from subjects with COPD (Opazo Saez et al., 2000). This proposal was without knowledge of whether integrin expression changes along with increased V_EC with within the ASM layer. If ECM were to increase without a change in coupling sites, then contractile response may not increase, as was the case in this study. Additional mechanistic probes are required to examine potential changes in integrin expression as a result of variation in
ECM, and while this is beyond the scope of the present study, integrins have been considered as a potential therapeutic target in obstructive disease (Wright et al., 2014), supporting the need for future study.

Another unresolved question is how changes in ECM would affect muscle shortening, which was not measured in the present study. Theoretically, increased ECM provides a mechanical opposition to shortening, and variation in ECM has been proposed as one reason why shortening capacity differs greatly between species (Opazo Saez et al., 2002). Dissolution of ECM within ASM strips through application of collagenase increases shortening capacity (Meiss, 1999). It therefore stands to reason that increased ECM may provide a greater elastic afterload opposing ASM shortening, even though it is difficult to specifically isolate changes in afterload from force transmission. Increased internal load provided by ECM may explain why Opazo Saez et al. demonstrated increased ASM stress but no change in shortening capacity (Opazo Saez et al., 2000). No study to date has examined the relationship between the proportion of ECM within the ASM layer and muscle shortening.

In addition to examining induced ASM contraction, we measured intrinsic ASM tone from the change in tension / stress produced by theophylline. All airways from all subjects studied exhibited intrinsic ASM tone, and on average was approximately ⅓ of the total stress induced to ACh or His. Intrinsic tone, which we loosely define as contraction of the ASM layer in the absence of an exogenous stimulus, is frequently observed in isolated human ASM (Wylam et al., 2012, Rabe et al., 1993, Ellis and Undem, 1994), and in numerous other species such as dogs (Hughes et al., 1975) and guinea-pigs (Taylor et al., 1984). Pharmacological determinants of intrinsic tone are varied and include changes in calcium sensitization pathways, endogenous histaminergic or cholinergic stimuli, release of leukotrienes or extracellular calcium leaking into the cells (Fox and Daniel, 1979, Wylam et al., 2012, Gazzola et al., 2016). Given that healthy individuals exhibit little to no response to inhaled bronchodilators, it is possible that intrinsic tone is limited to the in vitro environment. However,
conventional spirometry includes a deep inspiration which produces mechanical dilation prior to the assessment of expiratory flow, making it harder to detect tone in subjects with normal lung function (Crimi et al., 2002). Computed tomography avoids these issues and has been used to demonstrate ASM tone from expansion of the airway lumen to β-agonists, in both subjects with and without respiratory disease (Brown and Togias, 2016). There is little doubt that in vivo intrinsic ASM tone is at least partially mediated by firing of the vagus nerve (Canning, 2006), and that this mechanism will be disrupted in vitro since central connections are severed. We did not attempt to identify the source of intrinsic tone, though we note a transient relaxation to atropine, suggesting some contribution from parasympathetic nerve endings even in vitro.

The obvious implication of intrinsic tone is greater narrowing of the airway lumen and airflow limitation. There are alternatively potential benefits of intrinsic ASM tone as contraction increases airway stiffness or rigidity (Noble et al., 2007). Tiddens et al. recruited 31 subjects with varying degrees of COPD and showed that collapsibility of peripheral airways actually increased after administration of a β-agonist (Tiddens et al., 1999). That is, removal of intrinsic tone increased the susceptibility of the airway to collapse. Similarly, in immature porcine airway segments held in a compressive state (-15 cmH₂O transmural pressure), contraction of the ASM to parasympathetic stimulation produced paradoxical bronchodilation (McFawn and Mitchell, 1997b). Such studies support a protective role of intrinsic ASM tone.

The major finding of the present study was that muscle composition correlated with the magnitude of intrinsic stress. There was a positive correlation between VVASM and intrinsic stress, and a negative correlation between VVECM and intrinsic stress. These are on the surface logical changes since greater ECM means less contractile elements with the ASM layer (or alternative less VVOTHER). Other factors are clearly involved since a 10% change in ECM produced several fold differences in intrinsic stress. As discussed, intrinsic stress will be altered by the concentrations of endogenous stimuli and also
integrin expression that affects distribution of force throughout the layer (Zhang and Gunst, 2008). Repeated contractile activation during the adaptation protocol also increased intrinsic tone. The precise mechanism behind a relationship between intrinsic $V_{VECM}$ and intrinsic stress therefore remains unclear. Nonetheless, we are able to speculate on how an inverse relationship between intrinsic stress and $V_{VECM}$ will affect patient severity (Fig. 3.10B). Increased $V_{VECM}$ within the ASM layer in subjects with COPD will favour a reduction in intrinsic stress, and if tone does indeed contribute to mechanical stabilization of the peripheral airway wall, the loss of tone in COPD will favour collapse. The attractiveness of this working paradigm is that loss of intrinsic stress would favour a reduced response to inhaled bronchodilator, a defining feature of COPD (Figure 3.11).

**Figure 3.11: Working hypothesis on the relationship between volume fraction of extracellular matrix ($V_{VECM}$), intrinsic tone and airflow limitation.** Increased $V_{VECM}$ within the airway smooth muscle (ASM) layer reduces intrinsic ASM tone, leading to loss of wall stiffness (elastance), greater airway collapse and lung function impairment. The same mechanism could explain the apparent loss of response to inhaled bronchodilator in subjects with chronic obstructive pulmonary disease (COPD).

While the discussion above is focused on changes in peripheral airways, large airways also exhibit intrinsic tone (Ellis and Undem, 1994; Gazzola et al., 2016). In our own study on bronchial segments (Chapter 2), no relationship between intrinsic ASM tone and muscle layer composition was found.
The reason for the discrepancy between studies could be due to the nature of the volume measurement used to assess intrinsic tone in bronchial segments. Increased airway volume after theophylline was measured using a servo (pressure)-controlled motor syringe, but only during specified periods of the experimental protocol. It was therefore possible that intrinsic tone was influenced by variable contributions from the applied exogenous agonists. How tone, assessed from measures of force/stress relate to airway narrowing, is also unclear. These differences aside, the effect of intrinsic tone may be expressed differently in large airways which have cartilaginous support preventing collapse (Noble et al., 2002, Jiang and Stephens, 1990). Intrinsic tone in large airways will favour increased resistance (McFawn and Mitchell, 1997b) while the resultant airway stiffening potentially reduces the capacity of the airway to dilate to breathing manoeuvres such as deep inspiration (Noble et al., 2007) or pharmacological stimuli (Ansell et al., 2014). Under these conditions, loss of intrinsic tone may be beneficial at the level of the large airway. A last important consideration is how tone impacts anatomical dead space. Gazzola et al. demonstrated that ASM has a greater capacity to prevent distension rather than to shorten (Gazzola et al., 2016), raising the possibility that the physiological role of ASM is to minimise dead space.

An unexpected finding was the positive relationship between intrinsic and ASM stress induced to ACh. If stress produced by the ASM layer is fixed and linked with the state of muscle activation, partial activation in the presence of intrinsic muscle tone should reduce the change in ASM stress produced by an exogenous stimulus. Instead, intrinsic stress enhanced subsequent contractile response. These observations are consistent with the phenomenon of force adaptation (Bosse et al., 2009, Bosse et al., 2010, Lee-Gosselin et al., 2015, Pascoe et al., 2012a) where persisting tone optimises or exaggerates force adaptation. In vivo simulations of intrinsic tone demonstrate the capacity for force adaptation to exaggerate overall airway narrowing response, particularly in peripheral airways (Gazzola et al., 2017). Our present data is perhaps the first innate demonstration of force adaptation whereby inter-subject variations in intrinsic stress determined subsequent
contractile response. It is also interesting that the same relationship between intrinsic stress was not replicated when contraction was induced with His, possibly related to prior treatment with atropine which as discussed reduced intrinsic stress.

Experiments performed in this study demonstrate that the structural composition of the ASM layer in terms of proportion of ECM and ASM modifies intrinsic tone. These findings are potentially relevant to the pathophysiology of COPD where there is increased \( V_{\text{ECM}} \) within the ASM layer which we propose leads to collapse of peripheral airways and reduced response to conventional bronchodilators. This proposal requires testing using preparations of ASM from subjects with a COPD classification.
Chapter 4: Relationship between \textit{in vivo} airway responsiveness to methacholine and airway smooth muscle composition.

4.1 Introduction

Activation and contraction of the airway smooth muscle (ASM) layer produces airway narrowing and subsequently airflow limitation. Numerous physiological factors govern the magnitude of airway narrowing to inhaled contractile agonists including ASM force production (Lambert \textit{et al.}, 1993), epithelial integrity (Mitchell \textit{et al.}, 1993), geometry (Wagers \textit{et al.}, 2004) and both airway wall (Seow \textit{et al.}, 2000) and parenchymal after-loads (Macklem, 1991). With respect to ASM force production, the thickness of the ASM layer is critical (Noble \textit{et al.}, 2013) as well as length-tension properties (Lee-Gosselin \textit{et al.}, 2013), adaptive responses to muscle length (Bosse \textit{et al.}, 2008, Wang \textit{et al.}, 2001) and force (Bosse \textit{et al.}, 2009, Bosse \textit{et al.}, 2010) and innate or temporal contractile phenotype (Opazo Saez \textit{et al.}, 2000, Bosse, 2014, Wright \textit{et al.}, 2013). Aberrations in one or more of these physiological processes or properties may lead to an exaggerated bronchoconstrictor response, referred to as airway hyper-responsiveness (AHR), which is strongly associated with asthma (Woolcock \textit{et al.}, 1984) and is also observed in chronic obstructive pulmonary disease (COPD) (Postma and Kerstjens, 1998).

A number of studies have examined the contractility of ASM cells. Increased contractility might be thought of as increased mechanical performance of stimulated muscle in terms of force production and/or shortening (at a comparable after-load) for a given volume of ASM. The majority of studies have shown that there is little to no change in contractility in subjects with asthma (Pascoe \textit{et al.}, 2012b), suggesting that the increased narrowing observed in asthma is related simply to more muscle (Noble \textit{et al.}, 2013, Oliver \textit{et al.}, 2007). In comparison, airway narrowing capacity is increased in isolated bronchial segments from subjects with COPD without a change in ASM thickness (Chapter 2), and ASM stress is enhanced with reduced lung function (Opazo Saez \textit{et al.}, 2000). Moreover,
unlike asthma where the muscle layer comprises the same proportion of ASM cells and matrix as in non-asthmatic subjects (James et al., 2012), there is a greater proportion of extracellular matrix (ECM) in COPD. These observations support a change in ASM phenotype (structural and mechanical) in COPD rather than in asthma, which may bear some relationship to AHR observed in COPD.

The significance of increased ECM within the ASM layer to ASM mechanics remains unclear. In an earlier study (Chapter 3), we related volume fractions of ECM ($V_{ECM}$) and ASM ($V_{ASM}$) with stress in peripheral airway rings from human subjects. These parameters of muscle composition were not correlated with stress induced to acetylcholine or histamine, suggesting that structural changes within the ASM layer did not affect mechanical performance. On the other hand, an increase in $V_{ECM}$ within the ASM layer was associated with reduced ‘intrinsic tone’, the contractile tone present without exogenous stimulation. Loss of intrinsic tone could increase the propensity to airway collapse due to a fall in rigidity (Noble et al., 2007, Tiddens et al., 1999) or indirectly reduce ASM responsiveness by preventing ASM adaptation to force or length (Bosse et al., 2009, Gazzola et al., 2017). The direct and indirect effects of ECM within the ASM layer remain to be determined.

The aim of the present study was to determine how composition of the ASM layer determines in vivo responsiveness to bronchoconstrictor challenge. Patients undergoing lung resection surgery to remove tumours were recruited. Prior to surgery, post-bronchodilator lung function was performed to establish fixed airflow limitation and on a separate day subjects underwent methacholine (MCh) challenge. Large and small airways were acquired from post-operative tissue for assessment of $V_{ASM}$ and $V_{ECM}$ within the ASM layer. We hypothesised that composition of the ASM layer would predict in vivo responsiveness.
4.2 Methods

Recruitment and lung function

Subjects (n = 38) undergoing lung resection surgery (i.e., lobectomy) to remove tumours (except for one subject with scarring due to bronchiectasis localised to the lingular lobe), were recruited and consent was obtained to perform spirometry, bronchial challenge and collect lung tissue for the purposes of the study. Baseline (post-bronchodilator) lung function was measured using a wedge spirometer (Vitalograph, Buckingham, UK) according to the American Thoracic Society (ATS) criteria (ATS, 1995). Subjects were classified as having airflow limitation if they had a post-bronchodilator (3 puffs of salbutamol from a metered dose inhaler, 100 μg/puff) FEV<sub>1</sub>/FVC ratio < 0.7 and FEV<sub>1</sub> < 80% predicted. Reference equations for predicted values were from the 3<sup>rd</sup> National Health and Nutrition Examination Survey (Hankinson <i>et al.</i>, 1999). Subjects completed a respiratory health questionnaire including general information about any illnesses, medications used and smoking history. No subject reported a prior diagnosis of asthma.

On a separate day, subjects were asked to withhold bronchodilator therapy for bronchial challenge. Airway responsiveness was assessed by measuring changes in lung function induced by the inhalation of increasing doses of MCh (0.03 – 210 μmol) using the hand-held dosimeter method (Yan <i>et al.</i>, 1983), also according to the ATS criteria (Crapo <i>et al.</i>, 2000). Approval for the study was obtained from the Human Research Ethics Committees of Sir Charles Gairdner Hospital, Perth, Western Australia, Australia (Approval number 2015-53).

Airway morphometry

Post-operative lung tissue was fixed by formaldehyde instillation and inflation at a positive pressure of 25 cmH<sub>2</sub>O. Airway tissue used in the study was acquired away from any tumour. Central (lobar) airways were sampled as well as blocks of lung parenchyma (containing smaller airways) cut at random locations within the lobe. Airway and lung tissue were then processed and embedded into
wax blocks. From each block, transverse 4 μm sections and 0.5 μm sections were cut for staining with haematoxylin and eosin and the Masson’s trichrome technique, respectively (Jones et al., 2014) (Chapters 2 and 3).

Measurements included both airway wall dimensions and volume fractions of ASM layer components. Airway wall dimensions were measured using morphometric software (newCAST version 4.2.1, Visiopharm A/S, Horsholm, Denmark) and included the perimeter of the basement membrane (P_{bm}) and areas of the ASM, inner wall (W_{ai}), outer wall (W_{ao}) and total wall (W_{at}). Areas were square rooted and normalised to P_{bm}, correcting for airway size (James et al., 1988). The V_{VASM}, V_{VECM} and other components (V_{VOTHER}), i.e., spaces due to fixation artefact, inflammatory cells and blood vessels, within the ASM layer were measured on the 0.5 μm sections using point counting as previously described (Jones et al., 2014) (Chapters 2 and 3). Briefly, the ASM layer was outlined and then point grids were randomly generated by the software and projected, within the area outlined, on to the ASM layer, magnified at high power (×1000). The ASM layer was then randomly sampled around each transverse section until 200 points or more falling on the ASM, ECM or “Other” were counted.

Analysis and statistics

Airway responsiveness was defined by the provocative dose producing a 20% fall in FEV1 (PD_{20}). This was calculated by linear interpolation using the dose-response data immediately before and after an observed 20% fall. The PD_{20}(μmol) was log-transformed for statistical analyses. AHR was defined as a positive response to methacholine, specifically a PD_{20} of less than 4 μmol as previously described (Crapo et al., 2000). Dose-response slope (over the entire curve) was also calculated from the total percentage change in FEV1 divided by the total cumulative dose of MCh (% Δ FEV1 per μmol).
Airways were classified as large for $P_{bm} > 6$ mm and small for $P_{bm} < 6$ mm. Data were analysed and presented separately for large airways only (0-3/subject), small airways only (0-3/subject) or for all airways (large and small) combined.

Unpaired $t$-tests (with Welch’s correction or Mann-Whitney test, as appropriate) were used for comparisons between subjects with and without COPD. Sex-distribution was compared by chi-squared analysis. Correlations between morphological and physiological variables were determined using Pearson’s correlation coefficient or non-parametric Spearman’s correlation, depending on normality. Graphpad Prism (version 7.02; Graphpad Software) was used for data analysis and graphical representation. Data are mean ± standard error of the mean (SEM) with $p < 0.05$ as the level for statistical significance.

4.3 Results

Subject characteristics

Of the 38 subjects recruited, tissue was obtained from 22 subjects and these subjects were therefore included in the study. Patient demographics and lung function are shown in Table 4.1. Eight subjects had fixed airflow limitation i.e., post-bronchodilator FEV$_1 < 80\%$ of the predicted value and an FEV$_1$/FVC ratio < 0.7, and were therefore classified as having COPD. The remainder of subjects (n = 14) were assigned to the control group. There was no difference in subject age ($p = 0.82$) or sex distribution ($p = 0.14$) between groups. All subjects had a history of smoking with ten of the subjects being current smokers. No data were collected on past respiratory symptoms. Ten subjects were not using any medication, while the remainder were using a variety of anti-depressant and cardiovascular medications. None were using inhaled respiratory medications.

Out of the 22 subjects that underwent bronchial challenge, 17 exhibited at least a 20% fall in FEV$_1$ (control n = 10; COPD n = 7) allowing calculation of PD$_{20}$. One subject with COPD and 4 subjects
from the control group did not achieve a 20% fall in FEV₁. Dose-response slope was calculated for all subjects. There were no significant differences in PD₂₀ (p = 0.54) or dose-response slope (p = 0.40) between the control group and the COPD group. There was also no correlation between absolute FEV₁ (L) and PD₂₀ (r = 0.33, p = 0.20) or dose-response slope (r = -0.19, p = 0.40), respectively. Similarly, FEV₁/FVC was not correlated with PD₂₀ (r = 0.14, p = 0.60) or dose-response slope (r = -0.1, p = 0.67). There was no difference in the proportion of subjects with or without AHR in the COPD group (Positive 2; Negative 6) compared with the control group (Positive 4; Negative 10; Chi-squared = 0.18; p = 0.86).
Table 4.1: Subject demographics and lung function.

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<td>79</td>
<td>84</td>
<td>0.71</td>
</tr>
<tr>
<td>F</td>
<td>37</td>
<td>Current</td>
<td>101</td>
<td>102</td>
<td>0.82</td>
</tr>
<tr>
<td>M</td>
<td>63</td>
<td>Ex</td>
<td>106</td>
<td>116</td>
<td>0.69</td>
</tr>
<tr>
<td>M</td>
<td>62</td>
<td>Ex</td>
<td>83</td>
<td>107</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>64 ± 3</td>
<td>85 ± 3</td>
<td>86 ± 4</td>
<td>0.75 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

**COPD**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Smoking Status</th>
<th>FEV&lt;sub&gt;1&lt;/sub&gt; %pred</th>
<th>FVC %pred</th>
<th>FEV&lt;sub&gt;1&lt;/sub&gt;/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>78</td>
<td>Current</td>
<td>44</td>
<td>48</td>
<td>0.66</td>
</tr>
<tr>
<td>M</td>
<td>52</td>
<td>Current</td>
<td>72</td>
<td>83</td>
<td>0.66</td>
</tr>
<tr>
<td>M</td>
<td>46</td>
<td>Current</td>
<td>65</td>
<td>78</td>
<td>0.66</td>
</tr>
<tr>
<td>M</td>
<td>70</td>
<td>Ex</td>
<td>67</td>
<td>93</td>
<td>0.53</td>
</tr>
<tr>
<td>M</td>
<td>67</td>
<td>Ex</td>
<td>74</td>
<td>68</td>
<td>0.66</td>
</tr>
<tr>
<td>M</td>
<td>57</td>
<td>Current</td>
<td>76</td>
<td>90</td>
<td>0.65</td>
</tr>
<tr>
<td>M</td>
<td>67</td>
<td>Current</td>
<td>74</td>
<td>104</td>
<td>0.53</td>
</tr>
<tr>
<td>F</td>
<td>64</td>
<td>Ex</td>
<td>79</td>
<td>93</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>63 ± 4</td>
<td>69 ± 4</td>
<td>82 ± 6</td>
<td>0.63 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

M = male; F = female; % pred = percent predicted; Ex = ex-smoker; non = non-smoker. Bold text indicates average data including mean ± SEM where applicable.
Table 4.2: Subjects’ lung pathology and resected lobe location.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Tumour location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td></td>
</tr>
<tr>
<td>Metastatic hemangiopericytoma</td>
<td>LLL</td>
</tr>
<tr>
<td>Well-differentiated squamous cell carcinoma</td>
<td>LUL</td>
</tr>
<tr>
<td>Moderately well-differentiated squamous cell carcinoma</td>
<td>RUL</td>
</tr>
<tr>
<td>Metastatic colonic adenocarcinoma</td>
<td>LLL</td>
</tr>
<tr>
<td>Bronchiolo-alveolar cell carcinoma</td>
<td>LLL</td>
</tr>
<tr>
<td>Moderately poorly-differentiated adenocarcinoma</td>
<td>LUL</td>
</tr>
<tr>
<td>Moderately well-differentiated adenocarcinoma</td>
<td>RML</td>
</tr>
<tr>
<td>Moderately differentiated squamous cell carcinoma</td>
<td>LLL</td>
</tr>
<tr>
<td>Atypical carcinoid tumour</td>
<td>RLL</td>
</tr>
<tr>
<td>Moderately well-differentiated adenocarcinoma</td>
<td>RUL</td>
</tr>
<tr>
<td>Well-differentiated adenocarcinoma</td>
<td>LLL</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>Lingular lobe</td>
</tr>
<tr>
<td>Carcinoid tumour</td>
<td>RLL</td>
</tr>
<tr>
<td>Moderately well-differentiated adenocarcinoma</td>
<td>Lingular lobe</td>
</tr>
<tr>
<td><strong>COPD</strong></td>
<td></td>
</tr>
<tr>
<td>Poorly-differentiated squamous cell carcinoma</td>
<td>LUL</td>
</tr>
<tr>
<td>Poorly-differentiated adenocarcinoma</td>
<td>RUL</td>
</tr>
<tr>
<td>Moderately well-differentiated squamous cell carcinoma</td>
<td>RLL</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>RLL</td>
</tr>
<tr>
<td>Bronchio-alveolar carcinoma</td>
<td>LLL</td>
</tr>
<tr>
<td>Well-differentiated adenocarcinoma</td>
<td>RLL</td>
</tr>
<tr>
<td>Moderately well-differentiated squamous cell carcinoma</td>
<td>RLL</td>
</tr>
<tr>
<td>Moderately well-differentiated squamous cell carcinoma</td>
<td>RUL</td>
</tr>
</tbody>
</table>

LUL = left upper lobectomy; RUL = right upper lobectomy; LLL = left lower lobectomy; RLL = right lower lobectomy; RML = right middle lobectomy.

**Effect of composition of the ASM layer on lung function**

Data were analysed as case-means for all airways combined (control n = 14; COPD n = 8) or separately as large (control n = 10; COPD n = 7) or small (control n = 7; COPD n = 1) airways. Only 3 subjects had both large and small airways available, all of which were in the control group. The mean $P_{bm}$ of large airways in the control group was $7.7 \pm 0.6$ mm and $9.7 \pm 1.5$ mm in the COPD group ($p = 0.13$). The mean $P_{bm}$ of small airways was $5.3 \pm 0.2$ mm in the control group and $3.9$ mm in the COPD group.
When all airways were combined, $V_{VECM}$ was increased ($p = 0.04$) and $V_{VASM}$ was decreased ($p = 0.04$) in the COPD group compared with the control group (Table 4.3, Figure 4.1). There was no difference in $V_{VOTHER}$ between the groups ($p = 0.79$). After stratification of the data into large or small airways, due to the low numbers of small airways in the COPD group, only large airways were compared (Table 4.3, Figure 4.1). There was a non-significant trend (in the large airways) for an increase in $V_{VECM}$ ($p = 0.09$) and decrease in $V_{VASM}$ in the COPD group ($p = 0.05$). There was once again no difference in $V_{VOTHER}$ between groups ($p = 0.77$).

**Table 4.3: Composition of smooth muscle layer by subject group.**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>COPD</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large airways (n = 17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{VASM}$</td>
<td>0.55 ± 0.02</td>
<td>0.51 ± 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.28 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>$V_{VOTHER}$</td>
<td>0.17 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>All airways (n = 22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{VASM}$</td>
<td>0.55 ± 0.01</td>
<td>0.51 ± 0.01</td>
<td>*0.04</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.27 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>*0.04</td>
</tr>
<tr>
<td>$V_{VOTHER}$</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.79</td>
</tr>
</tbody>
</table>

$V_{VASM} =$ volume fraction of airway smooth muscle; $V_{VECM} =$ volume fraction of extracellular matrix; $V_{VOTHER} =$ volume fraction of “Other”. Small airways were not compared due to low numbers in the COPD group. * Significantly different compared with control ($p < 0.05$). Data are mean ± SEM.
Figure 4.1: Composition of the airway smooth muscle (ASM) layer in control subjects and in subjects with COPD. Data are plotted as large airways only (control n = 10; COPD n = 7) or as all large and small airways combined (control n = 14; COPD n = 8). A) there was a decrease in volume fraction of ASM ($V_{VASM}$) in COPD subjects when analysing all airways ($p = 0.04$) and a similar trend when large airways were analysed separately ($p = 0.05$); B) there was an increase in volume fraction of extracellular matrix ($V_{VECM}$) in the COPD group when analysing all airways combined ($p = 0.04$) and a similar trend when large airways were analysed separately ($p = 0.09$); C) no difference was observed in volume fraction of “Other” ($V_{VOTHER}$) in the COPD group when analysing all airways combined ($p = 0.79$) or when large airways were analysed separately ($p = 0.77$). Data are mean ± SEM.
Table 4.4: Correlations between lung function and composition of smooth muscle layer.

<table>
<thead>
<tr>
<th></th>
<th>FEV(_1) %pred</th>
<th>FEV(_1)/FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small airways (n = 8)</td>
<td>Large airways (n = 17)</td>
</tr>
<tr>
<td>(V_{VASM})</td>
<td>r = -0.06, p = 0.88</td>
<td>r = 0.07, p = 0.79</td>
</tr>
<tr>
<td>(V_{VECM})</td>
<td>r = -0.23, p = 0.57</td>
<td>r = -0.04, p = 0.87</td>
</tr>
<tr>
<td>(V_{VOTHER})</td>
<td>r = 0.36, p = 0.38</td>
<td>r = -0.04, p = 0.89</td>
</tr>
</tbody>
</table>

\(V_{VASM}\) = volume fraction of airway smooth muscle; \(V_{VECM}\) = volume fraction of extracellular matrix; \(V_{VOTHER}\) = volume fraction of “Other”. *Significant correlation (p < 0.05).

Correlations between pre-operative spirometry and tissue volume fractions within the ASM layer were largely non-significant (Table 4.4). The only statistically significant correlation was in small airways, where \(V_{VECM}\) was inversely related to FEV\(_1\)/FVC (Figure 4.2).
Figure 4.2: Correlations between FEV\textsubscript{1}/FVC and composition of the airway smooth muscle (ASM) layer in small airways. A) no correlation was found between volume fraction of ASM (V\textsubscript{VASM}) within the ASM layer and FEV\textsubscript{1}/FVC (r = 0.52, p = 0.19); B) a moderate inverse relationship was found between volume fraction of extracellular matrix (V\textsubscript{VECM}) and FEV\textsubscript{1}/FVC (r = -0.73, p = 0.04); C) no correlation was found between volume fraction of “Other” (V\textsubscript{OTHER}) and lung function (r = 0.28, p = 0.50; n = 18 for all correlations).

Composition of ASM layer was also compared between large (n = 17) and small airways (n = 8) when control and COPD cases were combined. There were no differences in V\textsubscript{VASM} (p = 0.15) and V\textsubscript{OTHER} (p = 0.62) between the large and small airways. There was a non-significant trend for increased V\textsubscript{VECM} in the large compared with small airways (p = 0.07).
Relation of the composition of the ASM layer to airway responsiveness

The results of correlative analyses between PD$_{20}$ and dose-response slope and volume fractions of the ASM layer are provided in Table 4.5. No significant correlations were found between dose-response slope and V$_{VASM}$, V$_{VECM}$ and V$_{OTHER}$ in the small airways, large airways or when combined (p > 0.05), although the positive relationship between V$_{VASM}$ in the large airways and dose-response slope was of borderline significance (r = 0.47, p = 0.06). There were also no statistically significant correlations between PD$_{20}$ and V$_{VASM}$, V$_{VECM}$ and V$_{OTHER}$ in the small airways (p > 0.05). There was however a significant inverse correlation between PD$_{20}$ and V$_{VASM}$ in the large airways (r = -0.57, p = 0.04), but not with V$_{VECM}$ and V$_{OTHER}$ (p > 0.05; Table 4.5, Figure 4.3).

Table 4.5: Correlations between composition of the ASM layer and airway responsiveness.

<table>
<thead>
<tr>
<th></th>
<th>Dose-response-slope</th>
<th>PD$_{20}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small airways</td>
<td>Large airways</td>
</tr>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 17)</td>
</tr>
<tr>
<td>V$_{VASM}$</td>
<td>r = -0.26</td>
<td>r = 0.47</td>
</tr>
<tr>
<td></td>
<td>p = 0.54</td>
<td>p = 0.06</td>
</tr>
<tr>
<td>V$_{VECM}$</td>
<td>r = 0.07</td>
<td>r = -0.28</td>
</tr>
<tr>
<td></td>
<td>p = 0.88</td>
<td>p = 0.27</td>
</tr>
<tr>
<td>V$_{OTHER}$</td>
<td>r = -0.14</td>
<td>r = -0.25</td>
</tr>
<tr>
<td></td>
<td>p = 0.75</td>
<td>p = 0.32</td>
</tr>
</tbody>
</table>

V$_{VASM}$ = volume fraction of airway smooth muscle; V$_{VECM}$ = volume fraction of extracellular matrix; V$_{OTHER}$ = volume fraction of Other”; PD$_{20}$ = provocative dose causing 20% fall in FEV$_1$; *Significant correlation (p < 0.05).
Figure 4.3: Correlation between provocative dose causing 20% fall in FEV₁ (PD₂₀) and composition of the airway smooth muscle (ASM) layer in large airways. A) an inverse correlation was observed with PD₂₀ and volume fraction of ASM (V_ASM) within the muscle layer (r = -0.57, p = 0.04); No correlations were found between PD₂₀ and; B) volume fraction of extracellular matrix (V_VECM) (r = 0.17, p = 0.58) and; C) volume fraction of “Other” (V_OTHER) (r = 0.45, p = 0.13; n = 13 for all correlations). PD₂₀ was logged for analyses.

Analyses were also performed separately in control subjects and COPD subjects. In control subjects, there was an inverse correlation between V_ASM in the large airways and PD₂₀ (r = -0.87, p = 0.02) and a positive correlation between V_OTHER in the large airways and PD₂₀ (r = 0.79, p = 0.04). When all airways were combined, a trend towards an inverse relationship between V_ASM and PD₂₀ was observed (r = -0.62, p = 0.06) as well as a trend towards a positive correlation between V_OTHER and PD₂₀ (r = 0.59, p = 0.07). Dose-response slope was not related to volume fractions within the ASM.
layer in control subjects. No correlations were observed between volume fractions within the ASM layer and PD$_{20}$ or dose-response slope in COPD subjects.

Comparisons between ASM composition in subjects with and without AHR are shown in Table 4.6. No differences in muscle composition were observed between subjects with and without AHR. Analyses were only performed using data derived from large airways and all airways combined (large and small airways) due to the low number of small airways available from subjects with AHR (n = 2).

Table 4.6: Muscle composition of subjects with and without AHR.

<table>
<thead>
<tr>
<th>Positive (&lt;4 μmol)</th>
<th>Negative (&gt;4 μmol)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{VASM}$</td>
<td>0.57 ± 0.02 (4)</td>
<td>0.54 ± 0.04 (2)</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.26 ± 0.01 (4)</td>
<td>0.24 ± 0.07 (2)</td>
</tr>
<tr>
<td>$V_{VOTHER}$</td>
<td>0.17 ± 0.03 (4)</td>
<td>0.21 ± 0.02 (2)</td>
</tr>
<tr>
<td>Small airways (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{VASM}$</td>
<td>0.57 ± 0.03 (4)</td>
<td>0.50 ± 0.01 (9)</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.29 ± 0.02 (4)</td>
<td>0.31 ± 0.02 (9)</td>
</tr>
<tr>
<td>$V_{VOTHER}$</td>
<td>0.14 ± 0.02 (4)</td>
<td>0.19 ± 0.02 (9)</td>
</tr>
<tr>
<td>Large airways (n = 13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{VASM}$</td>
<td>0.56 ± 0.02 (6)</td>
<td>0.51 ± 0.01 (11)</td>
</tr>
<tr>
<td>$V_{VECM}$</td>
<td>0.28 ± 0.02 (6)</td>
<td>0.30 ± 0.02 (11)</td>
</tr>
<tr>
<td>$V_{VOTHER}$</td>
<td>0.16 ± 0.02 (6)</td>
<td>0.19 ± 0.01 (11)</td>
</tr>
<tr>
<td>All airways (n = 17)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Airway hyper-responsiveness was identified by positive response to bronchial challenge. Positive and negative response to bronchial challenge was defined as a provocative dose that causes a 20% fall in FEV$_1$ of less than or greater than 4 μmol respectively; $V_{VASM}$ = volume fraction of airway smooth muscle; $V_{VECM}$ = volume fraction of extracellular matrix; $V_{VOTHER}$ = volume fraction of “Other”.

Small airways were not compared due to low numbers in the COPD group. Data are mean ± SEM. Brackets after mean ± SEM denotes number of positive or negative responders.
Effect of wall thickness on lung function and airway responsiveness

In large airways, significant inverse correlations were identified between FEV$_1$ %pred and √ASM/P$_{bm}$ ($r = -0.60$, $p = 0.01$), √W$_{ai}$/P$_{bm}$ ($r = -0.52$, $p = 0.03$), and √W$_{ao}$/P$_{bm}$ ($r = -0.51$, $p = 0.04$; Table 4.7; Figure 4.4). The only statistically significant correlation in the small airways was between √W$_{ai}$/P$_{bm}$ and FEV$_1$ %pred ($r = 0.74$, $p = 0.04$) where there was an unexpected positive correlation. No correlations were found between lung function and airway wall dimensions when all airways were combined.

Table 4.7: Correlative analysis between lung function and airway wall dimensions.

<table>
<thead>
<tr>
<th></th>
<th>Small airways</th>
<th>Large airways</th>
<th>All airways</th>
<th>Small airways</th>
<th>Large airways</th>
<th>All airways</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 17)</td>
<td>(n = 22)</td>
<td>(n = 8)</td>
<td>(n = 17)</td>
<td>(n = 22)</td>
</tr>
<tr>
<td>√ASM/P$_{bm}$</td>
<td>$r = 0.59$</td>
<td>$r = -0.60$</td>
<td>$r = -0.18$</td>
<td>$r = 0.56$</td>
<td>$r = -0.28$</td>
<td>$r = 0.10$</td>
</tr>
<tr>
<td>$p = 0.12$</td>
<td>$*p = 0.01$</td>
<td>$p = 0.42$</td>
<td>$p = 0.15$</td>
<td>$p = 0.27$</td>
<td>$p = 0.64$</td>
<td></td>
</tr>
<tr>
<td>√W$<em>{ai}$/P$</em>{bm}$</td>
<td>$r = 0.74$</td>
<td>$r = -0.12$</td>
<td>$r = -0.18$</td>
<td>$r = 0.68$</td>
<td>$r = -0.37$</td>
<td>$r = 0.10$</td>
</tr>
<tr>
<td>$*p = 0.04$</td>
<td>$p = 0.65$</td>
<td>$p = 0.42$</td>
<td>$p = 0.07$</td>
<td>$p = 0.14$</td>
<td>$p = 0.64$</td>
<td></td>
</tr>
<tr>
<td>√W$<em>{at}$/P$</em>{bm}$</td>
<td>$r = 0.20$</td>
<td>$r = -0.52$</td>
<td>$r = -0.33$</td>
<td>$r = 0.18$</td>
<td>$r = -0.47$</td>
<td>$r = -0.26$</td>
</tr>
<tr>
<td>$p = 0.64$</td>
<td>$*p = 0.03$</td>
<td>$p = 0.13$</td>
<td>$p = 0.67$</td>
<td>$p = 0.054$</td>
<td>$p = 0.24$</td>
<td></td>
</tr>
<tr>
<td>√W$<em>{ao}$/P$</em>{bm}$</td>
<td>$r = 0.14$</td>
<td>$r = -0.51$</td>
<td>$r = 0.35$</td>
<td>$r = 0.12$</td>
<td>$r = -0.48$</td>
<td>$r = -0.28$</td>
</tr>
<tr>
<td>$p = 0.74$</td>
<td>$*p = 0.04$</td>
<td>$p = 0.11$</td>
<td>$p = 0.77$</td>
<td>$*p = 0.048$</td>
<td>$p = 0.20$</td>
<td></td>
</tr>
</tbody>
</table>

√ASM/P$_{bm}$ = square root of airway smooth muscle normalized to perimeter basement membrane (P$_{bm}$); √W$_{ai}$/P$_{bm}$ = square root of inner wall area normalized to perimeter basement membrane; √W$_{at}$/P$_{bm}$ = square root of total wall area normalized to perimeter basement membrane; √W$_{ao}$/P$_{bm}$ = square root of outer wall area normalized to perimeter basement membrane; %pred = percent predicted. *Significant correlation ($p < 0.05$).
Figure 4.4: Correlations between lung function and airway wall dimensions in large airways. A) an inverse relationship was observed between FEV$_1$ %pred and muscle thickness ($\sqrt{\text{ASM}/P_{bm}}$; $r = -0.60$, $p = 0.01$); however, B) no correlation found between lung function and inner wall area ($\sqrt{\text{W}_{ai}/P_{bm}}$; $r = -0.12$, $p = 0.65$); C) an inverse relationship was observed between outer wall area ($\sqrt{\text{W}_{ao}/P_{bm}}$; $r = -0.51$, $p = 0.04$) as well as; D) total wall area ($\sqrt{\text{W}_{at}/P_{bm}}$) ($r = -0.52$, $p = 0.03$; $n = 17$).

Categorical data (Table 4.8) also showed an increase in $\sqrt{\text{W}_{at}/P_{bm}}$ ($p = 0.004$) and $\sqrt{\text{W}_{ao}/P_{bm}}$ ($p = 0.004$) in large airways from COPD subjects compared with control subjects. When all airways were combined, an increase was also found in $\sqrt{\text{W}_{at}/P_{bm}}$ ($p = 0.018$) and $\sqrt{\text{W}_{ao}/P_{bm}}$ ($p = 0.015$) in COPD subjects compared with control subjects.
Table 4.8: Airway wall dimensions between subject groups.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>COPD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large airways (n = 17)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>√ASM/P_{bm}</td>
<td>0.04 ± 0.003</td>
<td>0.05 ± 0.005</td>
<td>0.117</td>
</tr>
<tr>
<td>√W_{ai/P}_{bm}</td>
<td>0.11 ± 0.004</td>
<td>0.13 ± 0.007</td>
<td>0.103</td>
</tr>
<tr>
<td>√W_{at/P}_{bm}</td>
<td>0.30 ± 0.016</td>
<td>0.37 ± 0.013</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>√W_{ao/P}_{bm}</td>
<td>0.27 ± 0.015</td>
<td>0.34 ± 0.013</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td><strong>All airways (n = 22)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>√ASM/P_{bm}</td>
<td>0.05 ± 0.003</td>
<td>0.05 ± 0.005</td>
<td>0.538</td>
</tr>
<tr>
<td>√W_{ai/P}_{bm}</td>
<td>0.12 ± 0.004</td>
<td>0.12 ± 0.006</td>
<td>0.493</td>
</tr>
<tr>
<td>√W_{at/P}_{bm}</td>
<td>0.32 ± 0.015</td>
<td>0.37 ± 0.011</td>
<td><strong>0.018</strong></td>
</tr>
<tr>
<td>√W_{ao/P}_{bm}</td>
<td>0.29 ± 0.015</td>
<td>0.34 ± 0.012</td>
<td><strong>0.015</strong></td>
</tr>
</tbody>
</table>

√ASM/P_{bm} = square root of airway smooth muscle normalized to perimeter basement membrane (P_{bm}); √W_{ai/P}_{bm} = square root of inner wall area normalized to perimeter basement membrane; √W_{at/P}_{bm} = square root of total wall area normalized to perimeter basement membrane; √W_{ao/P}_{bm} = square root of outer wall area normalized to perimeter basement membrane. Small airways were not compared due to low numbers in the COPD group. * Significantly different compared with control (p < 0.05). **Significantly different compared with control (p < 0.01). Data are mean ± SEM.

Finally, we examined whether wall thickness was related to airway responsiveness. No correlations were found between PD_{20} and √ASM/P_{bm}, √W_{ai/P}_{bm}, √W_{at/P}_{bm}, or √W_{ao/P}_{bm} in the small, large airways or when all airways were combined (Table 4.9).
Table 4.9: Correlation between airway responsiveness and airway wall dimensions.

<table>
<thead>
<tr>
<th></th>
<th>Small airways</th>
<th>Large airways</th>
<th>All airways</th>
<th>Small airways</th>
<th>Large airways</th>
<th>All airways</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \sqrt{\text{ASM/P_{bm}}} )</td>
<td>( r = 0.33 )</td>
<td>( r = -0.34 )</td>
<td>( r = -0.10 )</td>
<td>( r = 0.37 )</td>
<td>( r = 0.44 )</td>
<td>( r = 0.15 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.43 )</td>
<td>( p = 0.18 )</td>
<td>( p = 0.69 )</td>
<td>( p = 0.49 )</td>
<td>( p = 0.13 )</td>
<td>( p = 0.56 )</td>
</tr>
<tr>
<td>( \sqrt{W_{ai}/P_{bm}} )</td>
<td>( r = -0.21 )</td>
<td>( r = -0.08 )</td>
<td>( r = -0.007 )</td>
<td>( r = 0.37 )</td>
<td>( r = 0.02 )</td>
<td>( r = -0.11 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.62 )</td>
<td>( p = 0.77 )</td>
<td>( p = 0.97 )</td>
<td>( p = 0.49 )</td>
<td>( p = 0.96 )</td>
<td>( p = 0.68 )</td>
</tr>
<tr>
<td>( \sqrt{W_{at}/P_{bm}} )</td>
<td>( r = 0.43 )</td>
<td>( r = 0.10 )</td>
<td>( r = 0.29 )</td>
<td>( r = 0.09 )</td>
<td>( r = 0.06 )</td>
<td>( r = -0.13 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.30 )</td>
<td>( p = 0.70 )</td>
<td>( p = 0.19 )</td>
<td>( p = 0.86 )</td>
<td>( p = 0.85 )</td>
<td>( p = 0.61 )</td>
</tr>
<tr>
<td>( \sqrt{W_{ao}/P_{bm}} )</td>
<td>( r = 0.52 )</td>
<td>( r = 0.16 )</td>
<td>( r = 0.32 )</td>
<td>( r = 0.05 )</td>
<td>( r = 0.05 )</td>
<td>( r = -0.14 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.20 )</td>
<td>( p = 0.55 )</td>
<td>( p = 0.14 )</td>
<td>( p = 0.93 )</td>
<td>( p = 0.86 )</td>
<td>( p = 0.60 )</td>
</tr>
</tbody>
</table>

\( PD_{20} \) = provocative dose causing 20% fall in FEV\(_1\); \( \sqrt{\text{ASM/P}_{bm}} \) = square root of airway smooth muscle normalized to perimeter basement membrane (P\(_{bm}\)); \( \sqrt{W_{ai}/P_{bm}} \) = square root of inner wall area normalized to P\(_{bm}\); \( \sqrt{W_{at}/P_{bm}} \) = square root of total wall area normalized to P\(_{bm}\); \( \sqrt{W_{ao}/P_{bm}} \) = square root of outer wall area normalized to P\(_{bm}\).

4.4 Discussion

Histological studies have demonstrated a shift in the composition of the ASM layer in subjects with fixed airflow limitation, specifically a disproportionate increase in ECM (Jones et al., 2016b). Whether this change in composition of the ASM layer is the cause of functional impairment is an area of ongoing investigation. This study assessed the relationship between in vivo bronchial responsiveness and composition of the ASM layer in subjects with and without fixed airflow limitation. In agreement with previous studies (Jones et al., 2016b) (Chapter 2), \( V_{VECM} \) was increased in the ASM layer of subjects with fixed airflow limitation, and there was an accompanying decrease in \( V_{VASM} \). While increased \( V_{VASM} \) was associated with a lower \( PD_{20} \) (i.e., increased responsiveness), there were no associations between \( V_{VECM} \) and airway responsiveness. Findings provide preliminary
evidence for a relationship between composition of the ASM layer and airway responsiveness that may affect lung function in subjects with COPD.

Assessing the relationship between ASM structure and lung function including airway responsiveness is difficult and to this day there are limited available methodologies. Computed tomography cannot visualise the ASM layer (Brown and Mitzner, 2003) and while optical coherence tomography shows promise, it is still in a developmental stage (Cho et al., 2016, Adams et al., 2016, Ansell et al., 2015). An alternative and well established approach is to assess lung function and/or response to bronchial challenge in vivo and to subsequently analyse structure on post-operative tissue (Gerrard et al., 1980). This post-operative histological approach has advantages compared with the very small tissue samples obtained at bronchoscopy biopsy (Lee et al., 2002, Laprise et al., 1999), since the entire airway wall can be examined in both small and large airways.

Large and small airways were obtained from post-operative specimens. The definition of a large versus small airway is somewhat ambiguous, but is traditionally defined as airways with a diameter of less than 2 mm (Hogg et al., 1968). In histological studies, P_{bm} is used as an index of airway size, which was the approach followed in the present study. P_{bm} is not significantly altered by induced bronchoconstriction or inflation (James et al., 1988), although some more recent studies suggest small changes with the latter (Noble et al., 2005b). Based on a cut-off for small airways of 6 mm P_{bm}, and assuming circularity, this equates to a predicted diameter of 2 mm. Data were analysed as the average of small and large combined (within a patient) and of large and small airways separately. Due to tumour location and availability of tissue, only three subjects had both small and large airways available which represents a sampling bias that potentially affects analyses for all airways combined.

With respect to V_{VECM} and V_{VASM} within the ASM layer, there is little to no effect of airway size (James et al., 2012) or distance along the length of an airway segment (Jones et al., 2014). The regional consistency of ASM layer volume fractions reduces potential sampling bias. In the case of
gross wall dimensions, measurements were analysed as \( \sqrt{\text{area}/P_{bm}} \), which normalises for differences in airway size, but does not control for proportionate differences between airways across anatomical locations. Under-sampling may therefore reduce our capacity to observe relationships between airway dimensions and functional outcomes.

Similarly, a small sample size and missing values (inability to acquire both small and large airways from the same subject) limits the power of the study and prevents more sophisticated analyses such as factorial ANOVA or MANOVA. For this reason, we used more basic statistical analyses with t-test and simple correlation although the number of tests performed may inflate the family wise type I error rate (false positive). As this study is an explorative study with limited power from a small sample size it was considered better to risk false positives than falsely reject real results. As such the results could be considered suggestive more than significant and should be confirmed in larger studies with greater power. Importantly the comparisons which have large alpha probabilities in this study, for example the correlation of \( V_{VOTHER} \) to baseline lung function, can be considered biologically unimportant.

Twenty-two subjects with a history of smoking, and importantly without a prior diagnosis of asthma, were recruited for this study. Eight of these subjects had fixed airflow limitation (i.e., post-bronchodilator \( FEV_1 < 80\% \) and \( FEV_1/FVC \) ratio \(< 0.7 \)) and were therefore classified as “COPD”. Bronchial challenges to MCh were performed prior to surgery and airway responsiveness was assessed from \( PD_{20} \) and dose-response slope. Results of these analyses showed no difference in airway responsiveness between subjects with and without COPD. The lack of apparent AHR in subjects with COPD is not necessarily surprising since the severity is less than in subjects with asthma (Du Toit et al., 1986) and is estimated to be present in only 1 in 2 subjects with COPD (Scichilone et al., 2006). Failure to demonstrate AHR in subjects with COPD did not prevent us from testing our
primary aim, since indices of airway responsiveness were used as continuous variables and plotted against ASM layer volume fractions.

Previous studies have demonstrated that in subjects with fixed airflow limitation, the $V_{\text{VECM}}$ within the ASM layer is increased (Jones et al., 2016b) (Chapter 2). While our sample size is less than the original study which reported data from 39 subjects (Jones et al., 2016b), the inverse relationship between $V_{\text{VECM}}$ and FEV$_1$ was again observed in this study. When all airways were combined, $V_{\text{VECM}}$ was increased and $V_{\text{VASM}}$ decreased in subjects with COPD compared with control subjects, and there was an inverse relationship between $V_{\text{VECM}}$ and FEV$_1$/FVC in the small airways. A change in composition of the ASM layer has therefore been related to fixed airflow limitation in three different cohorts, providing greater confidence that this a real and consistent change associated with fixed airflow limitation. The functional effects of these changes in the composition of the ASM layer was further examined in the present study.

The primary aim of the study was to determine how composition of the ASM layer affects airway responsiveness. We have previously proposed several potential effects of increased $V_{\text{VECM}}$ within the ASM layer (Chapter 2). Given that transmission of force throughout the ASM layer is dependent upon ASM cell to ECM communications through transmembrane integrins (Zhang and Gunst, 2008), increased ECM deposition is likely to affect ASM stress generation. Specifically, increased coupling between ASM and ECM components could allow forces initiated by individual ASM cells to be transmitted throughout the entire ASM layer with a greater mechanical efficiency. Alternatively, a concomitant decrease in ASM contractile elements ($V_{\text{VASM}}$) would be expected to attenuate ASM stress. Related changes in intrinsic ASM tone will further impact induced ASM stress, via postulated force adaptation (Chapter 3). Extrapolation of these hypothetical changes in ASM mechanics to the in vivo environment would lead to the prediction that either an increase or a decrease in airway responsiveness is feasible in response to changes in the composition of the ASM layer. Results of the
present study support the latter possibility since $V_{VASM}$ was inversely associated with $PD_{20}$ and $V_{VASM}$ was reduced in subjects with COPD. In comparison, there was no association between ASM thickness ($\sqrt{ASM/P_{br}}$) and airway responsiveness, suggesting that $V_{VASM}$ within the ASM layer, rather than the absolute thickness was more important in determining airway responsiveness to a bronchoconstricting stimulus.

Establishing the mechanisms of AHR in subjects with COPD and indeed its clinical significance is a complicated area of research. One issue is the known inverse correlation between FEV$_1$/FVC and $PD_{20}$ in subjects with COPD, which is not observed in asthma (Scichilone et al., 2006). This raises the possibility that the starting airway calibre is an important factor and that AHR may be geometrically mediated, since small changes in radius thereafter produce large increases in resistance. Moreover, since $PD_{20}$ and dose-response slope are calculated from the percentage change in FEV$_1$, there is an effect of normalisation; a given change in FEV$_1$ will be proportionally larger in COPD subjects, who by definition, have reduced baseline FEV$_1$. It has in fact been argued that absolute changes in FEV$_1$ in response to bronchial challenge (mL rather than percentage) are less for COPD subjects than in subjects with normal lung function (Jones et al., 2016a). Yet even after AHR is corrected for lung function, it remains a predictor for mortality in COPD, suggesting factors other than pure geometry may be at play i.e., ASM contractility.

Like the assessment of AHR in subjects with COPD, studies that more specifically examine ASM contraction present a complicated scenario of disease pathogenesis. In subjects with COPD, the maximal ASM stress in small peripheral airway rings (Opazo Saez et al., 2000) and maximal airway narrowing of large bronchial segments are increased (Chapter 2). These changes are consistent with AHR. Since $V_{VECM}$ is increased in the ASM layer in subjects with fixed airflow limitation, we have previously examined the relationships between tissue volume fractions within the ASM layer and ASM stress induced by exogenous agonists (Chapter 3). While no associations were observed, $V_{VECM}$
was inversely related to intrinsic ASM tone. Reduced intrinsic ASM tone in COPD may have dual and opposing effects on airflow. Loss of intrinsic ASM tone favours collapse of peripheral airways due to reduced airway stiffness (Tiddens et al., 1999). In contrast, ASM tone is of course targeted in the treatment of COPD, and even though bronchodilator therapy is less effective in these patients, the approach is driven by the intuitive assumption that reversal of ASM tone will reduce airway resistance. Finally, in the present study, an increase in $V_{VECM}$ was also accompanied by a reduction in $V_{VASM}$ in subjects with COPD. Importantly, the only statistically significant association between the composition of the ASM layer and airway responsiveness was the inverse relationship between $V_{VASM}$ and $PD_{20}$. The relative reduction of contractile elements within the ASM layer, further compounded by reduced capacity for force adaptation (Pascoe et al., 2012a) may therefore favour reduced ASM contraction.

In secondary analyses, the thickness of various airway wall compartments was correlated with lung function and airway responsiveness. In large airways, the thickness of the ASM layer, outer and total wall were inversely related with $FEV_1 \%$predicted. These observations are in broad agreement with much larger studies examining the relationship between wall thickness and lung function. In a study population of 3096 subjects, wall thickness in airways of 10 and 20 mm lumen perimeter assessed by computed tomography (i.e., large airways) was a strong independent predictor of airflow limitation (Patel et al., 2008). A separate study on post-surgical specimens from 72 patients showed an inverse association between inner wall thickness of cartilaginous airways and $FEV_1/FVC$ (Tiddens et al., 1995). Remodelling in the small airways is also an important feature of COPD. Inner, outer and total wall thickness of small airways is increased in smokers with airflow limitation (Bosken et al., 1990), but again using much larger samples than that possible in the present study (from 60 subjects) which affords greater statistical power. With respect to the relationship between airway responsiveness and wall thickness, the scenario is less clear. No association was found between the thickness of large airways and $PC_{20}$ in 40 subjects with a history of smoking (Mullen et al., 1986). The relationship
between wall thickness and airway responsiveness was stronger in smaller airways. The thickness of
the inner airway wall was shown to be inversely related with PC\textsubscript{20} in 21 subjects with a variable
history of smoking and incidence of emphysema (Finkelstein \textit{et al.}, 1995). While this observation
was not confirmed in a larger study on 77 smokers with mild-moderate airflow limitation (Riess \textit{et
al.}, 1996), inner, outer and total wall thickness of small airways were increased in subjects with lower
PC\textsubscript{20} after adjusting for the level of airflow limitation. Failure to demonstrate any associations
between wall thickness and airway responsiveness in the present study likely relates to low statistical
power.

A major assumption of the present study was that structural changes in a few small and large airways
would be able to predict a global response to an inhaled bronchoconstrictor agent, which is clearly an
oversimplification. It is well established that the response to bronchoconstrictor agents is
heterogeneous, even within the healthy lung (King \textit{et al.}, 2004, Dame Carroll \textit{et al.}, 2015, Tzeng \textit{et
al.}, 2009). In asthma, heterogeneity of airway remodelling has been observed between (Elliot \textit{et al.},
2015) and within patients (Pascoe \textit{et al.}, 2017), which may contribute to ventilation inhomogeneities
after bronchoconstriction (de Lange \textit{et al.}, 2007). Admittedly, ventilation is not a predictor for AHR
in subjects with COPD (Hardaker \textit{et al.}, 2013), and in this regard, is distinct from asthma.
Nonetheless, methodological issues relating to heterogeneity of remodelling events can only be
overcome by sampling more airways from a greater number of subjects, and by considering variation
of morphological outcomes in addition to means (Donovan, 2017).

The origin of AHR in COPD is complex and likely consists of geometric, tissue mechanical and
humoral factors. The present study examined for the first time whether ASM composition affects
response to inhaled bronchoconstrictor agents. It was shown that V\textsubscript{VASM} but not V\textsubscript{VECM} was inversely
related to PD\textsubscript{20}. We speculate that if in subjects with COPD, increased V\textsubscript{VECM} in the ASM layer is
accompanied by reduction in V\textsubscript{VASM}, this would favour reduced airway responsiveness due to the loss
or disruption of contractile elements and processes. Changes to the $V_{\text{VASM}}$ and $V_{\text{VECM}}$ within the ASM layer of subjects with COPD may therefore affect airway narrowing capacity and interact with other above factors to determine the net effect on expiratory airflow.
Chapter 5: Hyperinflation of bronchi *in vitro* impairs bronchodilation to simulated breathing and increases sensitivity to contractile activation.

5.1 Introduction

A pathophysiological feature of chronic obstructive pulmonary disease (COPD) is a reduced bronchodilatory response to deep inspiration (DI) (Scichilone *et al.*, 2008, Scichilone *et al.*, 2004). A normal bronchodilatory response to DI is considered an important mechanism maintaining airway calibre and the absence of this response contributes to airflow limitation in obstructive disease (Brusasco *et al.*, 1998, Scichilone *et al.*, 2008, Fairshter, 1985). Bronchodilation to DI is at least partly due to stretch of the airway smooth muscle (ASM) and a subsequent reduction in constrictor force (Shen *et al.*, 1997, Noble *et al.*, 2012). Consequently, any mechanism that reduces distension of the ASM layer during DI may reduce bronchodilation. For instance, attenuation of airway stretch due to loss of alveolar attachments reduces bronchodilation to DI (Scichilone *et al.*, 2005).

An alternative possible mechanism that may reduce stretch of the ASM and therefore bronchodilation to DI is lung hyperinflation, commonly observed in subjects with COPD (O’Donnell and Laveneziana, 2006, Sciurba, 2004). Two principle mechanisms produce hyperinflation in obstructive disease: static- and dynamic hyperinflation. Static hyperinflation is caused by a loss of elastic recoil pressure, which in turn elevates resting lung volumes. The more prevalent dynamic hyperinflation is caused by expiratory airflow limitation and results in gas trapping (Ferguson, 2006, Brusasco and Fitting, 1996, Pellegrino and Brusasco, 1997a). Dynamic hyperinflation theoretically shifts the airway to a stiffer region of the pressure-volume curve. That is, due to the non-linear pressure-volume relationship of the airway (Harris, 2005, Venegas *et al.*, 1998), as the lung and airway inflates the airway becomes stiffer and therefore more resistant to mechanical stretch produced by DI.
Previous studies demonstrate the importance of lung volume on airway compliance and the response to oscillatory load. Compliance of porcine airways at a transmural pressure (P_{tm}) corresponding to functional residual capacity (FRC, 5 cmH$_2$O) is greater than under conditions of hyperinflation (25 cmH$_2$O) and conversely less than when the airway is maintained under conditions mimicking deep exhalation (-5 cmH$_2$O) (Ansell et al., 2015). Harvey et al. (Harvey et al., 2013) showed that deflation of bovine airways to a P$_{tm}$ of 1 cmH$_2$O moved the airway towards a more compliant region of the pressure-volume curve and increased the bronchodilatory effect of pressure oscillation. Since a reduction in the effective P$_{tm}$ at FRC increases the response to pressure oscillations, we reasoned that an elevated P$_{tm}$ during hyperinflation would impair bronchodilation to DI.

The present study compared the effect of breathing manoeuvres applied at 5 cmH$_2$O (control) or 20 cmH$_2$O (hyperinflated) P$_{tm}$ in porcine bronchial segments. Active pressure to acetylcholine (ACh) was measured under static conditions and during simulated breathing to assess bronchodilation. We hypothesised that hyperinflation would reduce the bronchodilator response to breathing.

5.2 Methods

Animals

Castrated, white Landrace male pigs (~35 kg) were sedated with tiletamine-zolazepam (4.4 mg/kg i.m) and xylazine (2.2 mg/kg i.m.) and exsanguinated under sodium pentobarbitone anaesthesia (30 mg/kg). The lungs were removed and preserved on ice for dissection. The Institutional Ethics Committee and Animal Care Unit approved all animal handling and experiments.

Airway preparation

Bronchial segments from left upper lobes (2.0-3.5 mm diameter; ~40 mm in length) were dissected free from parenchyma and had all side-branches ligated with surgical silk. Proximal and distal diameters were measured by the delicate insertion of drill bits into the airway lumen. Segments were
cannulated and placed horizontally in organ bath chambers (custom-made; Figure 5.1) containing gassed (5% CO2:95% O2) Krebs solution (mM: NaCl 121; KCl 5.4; MgSO4 1.2; NaHCO3 25; sodium morpholinopropane sulphonic acid 5.0; glucose 11.5; and CaCl2 2.5) at 37°C (pH = 7.3). Bronchial segments were stretched to 105% of its dissected length to approximate the length at FRC in the porcine lung (Noble et al., 2005a) and remained at that length for the duration of the experiment. The opening of three-way taps at either side of the segment provided opportunity to flush the lumen with Krebs solution. During measurements, taps were closed to produce an isovolumetric state for measurement of active pressure to ACh, or to facilitate oscillation of airway lumen volume and pressure through movement of a motorised syringe plunger (Ansell et al., 2009a, Noble et al., 2007).

Figure 5.1: Schematic of the organ bath system used to study bronchial segments. Bronchial segments were cannulated and mounted in organ bath chambers (filled with gassed and heated Krebs solution) at either 5 cmH2O or 20 cmH2O transmural pressure (Ptm) set by the height of a hydrostatic pressure column. During measurements, a tap at one end of the segment (Tap 1) was rotated to create a closed environment. Under these conditions, contractile agonists produced an increase in intraluminal pressure (active pressure) measured by a transducer. A motorised syringe was used to produce volume oscillations. Between measurements, a second tap (Tap 2) was rotated to flush the airway lumen with Krebs solution.
As described below, bronchial segments were used to examine the effect of hyperinflation on the capacity for breathing manoeuvres to reverse ASM contraction (i.e., cause bronchodilation). The effect of hyperinflation on ASM contraction was further examined in bronchial rings. Porcine bronchial rings (2 mm diameter and length) were mounted in organ bath chambers of a DMT myograph system (620 M; Figure 5.2). Each bath was fitted with two mounting pins, one of which was connected to a micrometer that allowed the perimeter of the ring to be altered, and the second to a force transducer.

![Figure 5.2: Schematic of the organ bath system used to study bronchial rings.](image)

Bronchial rings were mounted in organ bath chambers of a DMT myograph system (620M) filled with gassed and heated Krebs solution. Rings were inserted over a pair of stainless steel mounting pins. One mounting pin was connected to a micrometer and the other pin to a force transducer. All rings were initially stretched to a tension of 0.2 g and the linear distance between the external surface of the mounting pins was defined as $L_{ref}$ (reference length). A) Rings were either maintained at $L_{ref}$ or B) stretched to a length 1.3 times that of $L_{ref}$ (1.3$L_{ref}$), as described in the main text.

**Bronchial segment protocol**

Once in the organ bath, bronchi underwent assessment for leaks by transiently inflating the airway with a dye to ~50 cmH₂O Pₜₐₘ. Bronchi were equilibrated in the organ bath to either 5 cmH₂O Pₜₐₘ (control state; n = 8) or 20 cmH₂O Pₜₐₘ (hyperinflated state; n = 10) for ~2 hours. After 1 h, viability of the tissue was confirmed by contraction to ACh (10⁻⁴ M), followed by a 30 min recovery period.
To assess the effects of inflation state (control vs. hyperinflated) on contractile responses to simulated breathing, cumulative dose-response curves (DRCs) to ACh (10^{-7} M to 10^{-2} M) were performed under static conditions and during volume oscillation (and therefore P_{tm}). Oscillation was achieved through the motorised movement of a syringe plunger that produced cyclical changes in lumen volume (Noble et al., 2007). Volume oscillations were adjusted in both groups to produce a 10 cmH2O trough-to-peak change in P_{tm} for the relaxed airway wall. That is, control bronchi were cycled from 5 to 15 cmH2O P_{tm} and hyperinflated bronchi were cycled from 20 to 30 cmH2O P_{tm}.

Recordings were performed in a closed system under isovolumetric conditions or during simulated breathing. Intraluminal pressure (and therefore P_{tm}) was measured by a calibrated transducer (Gould, model P23ID) and PowerLab data-acquisition system (ADInstruments). Airway contraction (active pressure) to ACh was assessed from the increase in intraluminal pressure under isovolumic conditions or from changes in trough pressures during volume oscillation as previously described (Noble et al., 2007). Stiffness was measured from the trough-to-peak pressure amplitude and the previously determined volume oscillation (i.e., cmH2O/μL).

**Bronchial ring protocol**

After mounting in the organ bath, bronchial rings were stretched to a pre-load of 0.2 g. The linear distance between the external surface of the mounting pins was measured (reference length, L_{ref}) which is equal to approximately half lumen perimeter (Chapter 3). L_{ref} was determined by the micrometer reading (DMT myograph system, 620M), which had previously been calibrated to absolute distance using a dissecting microscope and 1 mm graticule. The width of the ring was also measured under the dissecting microscope, which was used for normalization of force, see below.

One group of bronchial rings were maintained at L_{ref} (control; n = 4) for the first part of the experiment and then stretched to 1.3L_{ref} for the remainder of the experiment. A second set of rings were
immediately stretched to 1.3L\textsubscript{ref} (hyperinflated; n = 4) and then shortened to L\textsubscript{ref} for the remainder of the experiment. This approach controlled for any order bias and when data was combined provided a group size of 8 control and 8 hyperinflated-paired experiments. Bronchial rings (at L\textsubscript{ref} or 1.3L\textsubscript{ref}) were allowed to equilibrate for \~1 h, before tissue viability was confirmed to ACh (10^{-4} M). A period of ‘length-adaptation’ followed (Bosse \textit{et al.}, 2008, Wang \textit{et al.}, 2001), which comprised of repeated isometric contractions to KCl (60 mM). Each ring received six contractions separated by 5 min rest periods over a total period of approximately 2 h.

At the end of the adaptation period, dose-response curves to ACh (10^{-8} M – 3 \times 10^{-3} M) were constructed at L\textsubscript{ref} and 1.3L\textsubscript{ref}. Once ACh was fully washed from the tissue and airway contraction reversed, rings were moved to the alternative condition (L\textsubscript{ref} or 1.3L\textsubscript{ref}), adapted to a further 6 KCl stimulations and a second ACh dose-response curve was performed. Force (g) measurements were calculated from the calibrated force transducer and normalized to the width of each ring (i.e., tension in g/mm). Since force recordings in ring preparations are effectively double that present in an intact bronchial segment (i.e., both sides of the ring contribute to the measured force), recorded active and passive forces were halved.

\textit{Analysis and statistics}

Sigmoidal dose-response curves (variable slope) were fit to both the pressure and tension data to compute both \(E_{max}\) (maximal response) and pD\textsubscript{2} (negative logarithm of dose producing half maximal response) parameters. \(E_{max}\) and pD\textsubscript{2} in bronchial segments were compared using two-way ANOVA with inflation state as a non-repeat measures variable, and oscillation (static v oscillation) as a repeat measures variable. Stiffness (cmH\textsubscript{2}O/\mu L) was also compared using two-way ANOVA with dose as a repeat-measure variable. Differences in \(E_{max}\) and pD\textsubscript{2} between control (L\textsubscript{ref}) and hyperinflated rings (1.3L\textsubscript{ref}) were assessed using a Student’s paired \(t\)-test. GraphPad Prism (version 7.02; Graphpad
Software) was used for analysis. Data are mean ± standard error of the mean (SEM) with p < 0.05 as the level for statistical significance and n is the number of animals.

5.3 Results

Hyperinflation in bronchial segments

DRCs for control (n = 8) and hyperinflated (n = 10) bronchial segments under static and oscillatory conditions are shown in Figure 5.3, which were fitted with sigmoidal curves to determine $E_{\text{max}}$ and $pD_2$. $E_{\text{max}}$ in the hyperinflated segments was reduced under static conditions compared with control (p < 0.001). In contrast, there was an unexpected increase in $pD_2$ in hyperinflated segments compared with control (p < 0.05).

The response to breathing differed between control and hyperinflated bronchial segments. In control segments, oscillatory loads halved $E_{\text{max}}$ from 61.0 ± 3.8 to 29.7 ± 4.4 cmH$_2$O (p < 0.0001; Figure 5.4). In contrast, oscillation only reduced the $E_{\text{max}}$ of hyperinflated segments from 40.0 ± 2.5 to 31.2 ± 2.4 cmH$_2$O (p < 0.001 interaction; p < 0.05). The mean decrease in active pressure with oscillation was reduced from 50.1 ± 7.2% in control segments to 22.4 ± 2.5% in the hyperinflated segments (p < 0.01).
Figure 5.3: Dose-response curves to acetylcholine (ACh) performed in bronchial segments. A) control (transmural pressure = 5 cmH₂O; n = 8); and B) hyperinflated (transmural pressure = 20 cmH₂O; n = 10) bronchial segments under static conditions and during volume oscillations simulating breathing. Contractile response was assessed from increased lumen pressure i.e., active pressure. Static (filled circles); Oscillation (open circles). Data are mean ± SEM.

Due to greater bronchodilation to oscillation in the control bronchial segments, differences in $E_{\text{max}}$ between control and hyperinflated segments observed under static conditions were abolished in the presence of oscillation. Sensitivity was however not altered by oscillation for either control or hyperinflated segments, and hence hyperinflated segments under oscillation were also more sensitive than control segments under oscillation ($p < 0.05$).
Figure 5.4: Maximal response ($E_{max}$) and airway sensitivity ($pD_2$) of control and hyperinflated bronchial segments under static conditions and during volume oscillations simulating breathing. A) $E_{max}$ was greater in control ($n = 8$) compared with hyperinflated ($n = 10$) segments under static conditions. Oscillation reduced $E_{max}$ of both control and hyperinflated segments but by a greater extent in the control group ($p < 0.001$ interaction); B) $pD_2$ was increased in hyperinflated compared with control segments which was not altered by oscillation. *Significantly different from static ($p < 0.05$). #Significantly different from control ($p < 0.05$). Static (black bar); Oscillation (white bar). Data are mean ± SEM.

Airway wall stiffness was determined during oscillation in both groups in their relaxed and contracted state. In the relaxed state (prior to ACh), the stiffness in the control bronchial segments was considerably less ($1.4 \pm 0.3$ cmH$_2$O/μL) than in the hyperinflated segments ($3.3 \pm 0.4$ cmH$_2$O/μL; $p < 0.01$; unpaired t-test). However, following contraction with ACh, there was a greater increase in stiffness in control compared with hyperinflated segments (interaction $p < 0.01$; Figure 5.5). Mean stiffness calculated across the DRC (from relaxed state to maximal ACh dose) was however still less in control ($4.2 \pm 0.4$ cmH$_2$O/μL) compared with hyperinflated segments ($5.8 \pm 0.6$ cmH$_2$O/μL; $p < 0.05$).
Figure 5.5: Stiffness (cmH$_2$O/μL) of bronchial segments in the presence of acetylcholine (ACh).

There was partial convergence between control (transmural pressure = 5 cmH$_2$O; n = 8) and hyperinflation (transmural pressure = 20 cmH$_2$O; n = 10) curves with increasing ACh dose. *Significantly different from control (p < 0.05); Static (filled circles); Oscillation (open circles). Data are mean ± SEM.

Hyperinflation in bronchial rings

The increased sensitivity of hyperinflated bronchial segments described above suggests that changes in ASM length alter sensitivity. To provide supporting evidence of a change in sensitivity with hyperinflation, active tension was measured in bronchial rings studied at $L_{\text{ref}}$ and after 30% stretch ($1.3L_{\text{ref}}$; Figure 5.6). Sensitivity (pD$_2$) increased from 5.1 ± 0.13 to 5.5 ± 0.14 (p < 0.01; n = 8) at $L_{\text{ref}}$ and $1.3L_{\text{ref}}$ respectively. $E_{\text{max}}$ to ACh also increased from 0.30 ± 0.03 g/mm to 0.36 ± 0.03 g/mm, when length was increased from $L_{\text{ref}}$ to $1.3L_{\text{ref}}$ (p < 0.05; n = 8). In a separate group of bronchial rings (n = 8), the effect of order (1$^{\text{st}}$ or 2$^{\text{nd}}$ DRC) was assessed at either $L_{\text{ref}}$ or $1.3L_{\text{ref}}$. $E_{\text{max}}$ was modestly (~5%, p < 0.05) greater during the second DRC but there was no effect on sensitivity. As indicated in the methods, the primary protocols ($L_{\text{ref}}$ or $1.3L_{\text{ref}}$) were randomised to prevent any order bias.
Figure 5.6: Tension dose-response curves to acetylcholine (ACh). Bronchial rings were contracted under low pre-load and thus short smooth muscle length ($L_{ref}$; $n = 8$) or at a length 1.3 times $L_{ref}$ ($1.3L_{ref}$; $n = 8$). Contraction was assessed from active force generation (g), which was then normalized to the width of each bronchial ring (i.e., tension in g/mm). $L_{ref}$(filled circles); $1.3L_{ref}$(open circles). Data are mean ± SEM.

5.4 Discussion

Hyperinflation (O'Donnell and Laveneziana, 2006) and reduced bronchodilatory response to DI (Scichilone et al., 2004) are both physiological abnormalities in COPD. This study examined whether there is any causal relationship between these abnormalities. A bronchial tube model was used to show that hyperinflation inhibits the bronchodilation produced by simulated breathing. We propose that stiffening of the airway as a result of hyperinflation reduces distension of the ASM during breathing and in turn bronchodilation. Increased sensitivity to contractile activation after hyperinflation was also observed and along with reduced bronchodilation to breathing is expected to contribute to airflow limitation in obstructive disease.

Before discussing the implications of the present data to human health, methodological assumptions and limitations need to be considered. Bronchoconstrictor responses were examined in a single generation of a large airway which is a simplified model compared with integrated respiratory
function *in vivo*. Moreover, the airway response to mechanical stress and/or strain *in vivo* is demonstrated by transient responses to DI. We chose to instead assess the effects of hyperinflation on bronchodilation to a continuous oscillatory protocol equivalent to an amplitude of twice a normal tidal breath. Previous studies have shown that when volume oscillation is held fixed to that producing a $\Delta 5 \text{cmH}_2\text{O } P_{tm}$ in the relaxed airway, modest bronchodilation is observed (Ansell *et al.*, 2009b, Noble *et al.*, 2007) but which is greatly increased when amplitude is raised to twice tidal (i.e., $\Delta 10 \text{cmH}_2\text{O } P_{tm}$) (Ansell *et al.*, 2009b). For this reason, oscillation was applied at an amplitude of twice tidal to produce a robust bronchodilator response, thereby allowing any effect of hyperinflation to be delineated. With respect to confirmatory studies using bronchial rings, the hyperinflated state corresponded to a length 30% greater than the control state, which is somewhat arbitrary. This amplitude of stretch was chosen as this is at the upper end of the expected change in ASM length with lung inflation (~20-25%) (Noble *et al.*, 2004, Fredberg *et al.*, 1997) and therefore represented a maximal effect of ASM length change on sensitivity.

Bronchodilation to DI has been proposed as an innate mechanism maintaining airway patency. There is strong evidence that bronchodilation to respiratory manoeuvres is mediated by distension of the ASM. Length oscillation of isolated ASM reduces active force relative to static conditions (Gunst, 1983) and in bronchial segments, bronchodilation to simulated DI is in proportion to the amplitude of ASM stretch (Ansell *et al.*, 2013). An increase in airway stiffness in obstructive disease as a result of airway remodelling or enhanced muscle tone (Ward *et al.*, 2001, Baldi *et al.*, 2010) may therefore contribute to the loss of the beneficial response to DI by restricting transient increases in ASM length in response to fluctuations in $P_{tm}$ (Noble *et al.*, 2007). The present study suggests that mechanisms other than structural remodelling or muscle tone, notably lung hyperinflation, contributes to both an increase in airway stiffness and reduced bronchodilation to breathing manoeuvres.
It has been shown that reducing airway inflation below FRC increases the effectiveness of oscillatory loads at relaxing bronchi (Harvey et al., 2013), attributed to greater compliance of the deflated airway. The present study extended these findings by subjecting bronchi to oscillations mimicking breathing movements at an elevated FRC, such as that produced by dynamic hyperinflation. We suggest that the same phenomenon underlying increased response to oscillatory load at low lung volume also explains the reduced effectiveness of breathing in hyperinflated bronchi. That is, during normal breathing around 5 cmH$_2$O $P_{tm}$, the airways are more compliant, compared with higher distending pressures where there is pronounced stiffening (Harris, 2005, Noble et al., 2007) and a lower volume oscillation and in turn amplitude of ASM stretch. As a result of the reduction in the amplitude of oscillatory ASM stretch in hyperinflated airways, the bronchodilatory effect of breathing is attenuated.

While in the relaxed state, hyperinflated bronchial segments were stiffer than control; much of the increased stiffness of hyperinflated bronchi was abolished when the tissues were contracted. Classical pressure-volume compliance curves (Harris, 2005) have typically been performed on relaxed bronchi. Fewer studies have measured compliance curves in contracted airways, although those that have demonstrate a similar non-linearity of the pressure-volume curves (Gunst et al., 1990, Olsen et al., 1967, Harvey et al., 2013). Contraction of the ASM increases airway stiffness particularly at low lung volumes (Kelly et al., 2012). We have previously shown that at high $P_{tm}$ (25 cmH$_2$O) the effect of ASM contraction on increasing airway stiffness is diminished compared with lower $P_{tm}$ (5 cmH$_2$O) (Ansell et al., 2015), indicating that there is interdependence between the loading of active and passive elements within the ASM layer. A greater effect of ASM contraction on airway stiffness at low $P_{tm}$ compared with high $P_{tm}$ likely explains the partial convergence of the stiffness curves between control and hyperinflated segments. On the surface it may be surprising that hyperinflation suppressed breathing-induced bronchodilation of contracted bronchi when the stiffness became similar between hyperinflated and control bronchi after contraction. Importantly, the amplitude of
volume oscillation was pre-set to the compliance of the relaxed airway and was not affected by the change in stiffness brought about by the addition of ACh. Nonetheless, the stiffness of the hyperinflated bronchi averaged over the course of the DRC was greater compared with control and findings are therefore physiologically relevant.

It has recently been shown that elongation of the ASM increases contractile capacity (Gazzola et al., 2016), implicating lung hyperinflation as a potential mechanism producing airway hyper-responsiveness (AHR). Using ovine tracheal strips and human bronchial rings, Lee-Gosselin et al. (Lee-Gosselin et al., 2013) demonstrated that lengthening of the ASM increased both maximal response and sensitivity to bronchoconstrictor agonists, two primary characteristics of AHR (Woolcock et al., 1984). Although the present study confirms an increase in airway sensitivity as a result of hyperinflation (discussed below), maximal response was attenuated in bronchial segments by hyperinflation and augmented in bronchial rings. These conflicting observations can be explained by the different loading conditions between airway preparations. In bronchial segments, $P_{tm}$ is directly controlled by altering luminal pressure and as such, changes in maximal response are more representative of how the airway behaves in vivo. In the porcine airway, increased $P_{tm}$ above 5 cmH$_2$O reduces airway narrowing capacity and ASM tension calculated from active pressure and radius using the Laplace equation (Ansell et al., 2015). In comparison, bronchial rings were arbitrarily loaded with 0.2 g of force ($L_{ref}$) and compared with tissues conditioned to a length 1.3 times the reference length (1.3$L_{ref}$). This approach was appropriate since the aim of the experiment was to assess the effects of altered ASM length on airway sensitivity. The Laplace equation was again used to calculate effective $P_{tm}$ from passive force and ring width (Mulvany and Halpern, 1977) and was estimated to be ~2 and 5 cmH$_2$O at $L_{ref}$ and 1.3$L_{ref}$ respectively. Since contractile response is reduced at $P_{tm}$s below 5 cmH$_2$O in the porcine bronchus (Ansell et al., 2015), an increase in maximal response at 1.3$L_{ref}$ compared with $L_{ref}$ is expected as this positions the ASM closer to relative optimal length.
Hyperinflated bronchi were more sensitive to contractile agonists compared with control, consistent with Lee-Gosselin et al. (Lee-Gosselin et al., 2013) who showed that longer tracheal strips were more sensitive. We further explored the effect of ASM length in bronchial rings and again observed an increase in ASM sensitivity with greater ASM length. Three different preparations (bronchial tubes and rings, and tracheal strips) have therefore been used to demonstrate an increase in airway sensitivity with inflation, suggesting it’s a real phenomenon. Furthermore, increased sensitivity with hyperinflation was also observed using the oscillation protocol, indicating that these findings are relevant to the dynamic in vivo environment and are not impacted by transient airway stretch. The mechanism underlying the increase in sensitivity with inflation is unclear, but may involve various mechanotransduction pathways which modulate in ASM intracellular calcium concentration (Ito et al., 2008), ATP release (Takahara et al., 2014) and myosin phosphorylation and phosphatidylinositol turnover (Yoo et al., 1994).

Finally, any association between lung hyperinflation and AHR is dependent on relative changes in airway sensitivity and maximal response which both characterise AHR (Woolcock et al., 1991), as well as static opposed to dynamic airway properties. The results of Lee-Gosselin et al. (Lee-Gosselin et al., 2013) support an important role of hyperinflation in mediating AHR through changes in ASM force (maximal response and sensitivity), but this model does not consider possible changes in the response to dynamic load associated with breathing. Our study, while also showing an increase in airway sensitivity with hyperinflation, supports a reduction in maximal airway response under static conditions. These differences were however abolished in the presence of simulated breathing since there was significant bronchodilation in the control but not hyperinflated bronchi. The net effect of hyperinflation on maximal bronchoconstriction is therefore a balance between hyperinflation-dependent increases in ASM force and reduced bronchodilation to breathing manoeuvres.
In summary, this study shows that physiologically relevant levels of hyperinflation blunts bronchodilation to simulated breathing and causes a length-dependent change in ASM sensitivity. We propose that hyperinflation stiffens the airway wall and reduces distension of the ASM during DI and therefore the pursuing bronchodilation, and together with increased airway sensitivity contributes to airflow limitation in obstructive disease.
Chapter 6: Final discussion

The present thesis used a physiological approach to better understand the mechanisms contributing to the onset of fixed airflow limitation, the functional impairment that leads to a diagnosis or classification of chronic obstructive pulmonary disease (COPD). Concepts that were considered included remodelling of small and large airways, airway narrowing capacity, force generation of the airway smooth muscle (ASM) layer and the critical importance of the static and dynamic mechanical environment of the lung to airway function. This final discussion will now further articulate (with the freedom to be a little more speculative) how the results generated from these experiments have advanced our understanding on the pathogenesis of COPD and will also make recommendations for areas of future study.

Perhaps the most significant outcome of the project was that narrowing capacity of the large airway is increased in subjects with COPD (Chapter 2). Increased airway narrowing in vitro, is consistent with airway hyper-responsiveness (AHR) in vivo (Postma and Kerstjens, 1998), and also greater contractility of the ASM layer from subjects with COPD (Opazo Saez et al., 2000). This belies the apparent controversy surrounding AHR in COPD which is at times only attributed to a geometric effect of a reduced starting lung function (Scichilone et al., 2006) or issues relating to normalising changes measured after bronchial challenge to that lower lung function (Jones et al., 2016a). To the contrary, the extent of the change in airway narrowing capacity demonstrated in vitro, compares well with similar observations reported using bronchial segments acquired from subjects with a history of asthma (Noble et al., 2013). The assessment of airway narrowing in bronchial segments is therefore a proven approach that is able to adequately simulate the in vivo environment, unlike other methods and tissue preparations where in vitro airway responsiveness does not correlate well with in vivo airway responsiveness (Armour et al., 1984). The question then turns to why similar changes in airway narrowing capacity present as a fixed phenotype and variable phenotype in COPD and asthma respectively.
Could the above conundrum be related to phenotypic disparity in ASM structure-function? Clear differences are already evident in the behaviour of the ASM layer between COPD and asthma. Increased narrowing capacity of bronchial segments from subjects with COPD was observed without a change in ASM thickness, compared with asthma where the ASM layer is thickened and positively correlated with narrowing (Noble et al., 2013). Stress produced by the ASM layer is however increased in COPD (Opazo Saez et al., 2000) compared with asthma where most studies report no change (Ijpma et al., 2015, Chin et al., 2012). And of course, as clearly outlined in this thesis, there is expansion of extracellular matrix (ECM) components within the ASM layer from subjects with COPD (Jones et al., 2016b) (Chapters 2 and 4), and in that regard the disease is different to both subjects with asthma, and indeed individuals without respiratory impairment. One consideration is that increased volume fraction of ECM \( V_{VECM} \) within the ASM layer contributes to fixed rather than variable airflow limitation. While we had speculated that contractility of the ASM layer may be increased as a result of this compositional shift in the structure of the ASM layer (a proposal not necessarily supported by the data, see below), changes in the coupling between ASM cells and ECM may favour reduced relaxation of the shortened ASM layer. That is, exaggerated narrowing of the airway produced by exaggerated shortening of the ASM layer is essentially locked-down by greater ECM-ASM adhesions (Irons et al., 2018) present within the ASM of COPD subjects, producing irreversible airway narrowing. New data is therefore required on integrin expression and its relationship to changes in \( V_{VECM} \) within the ASM layer of COPD subjects.

The focus of the project was subsequently turned to the impact of composition of the ASM layer to force generation in small peripheral airways. Unfortunately, tissue from very few subjects with COPD could be recruited, despite obtaining consent from 90 subjects. Timing of surgeries became an increasingly significant obstacle in obtaining fresh human airway tissue from pathologists. Hence experiments performed in Chapter 3 related ASM composition to force production in a cohort with mostly normal lung function (despite lung cancer) and relied on natural biological variability.
Nonetheless, we reasoned that in view of known increases in ASM contractility from subjects with COPD (Opazo Saez et al., 2000), increased $V_{VECM}$ would positively relate to stress-induced by conventional contractile agonists. No such relationship was however observed, and overall composition of the ASM layer did not affect induced contractile response. In fact, based on our findings that intrinsic ASM tone is reduced by higher $V_{VECM}$ and lower $V_{VASM}$, the concept of force-adaptation (Bosse et al., 2009) could be evoked to propose a reduced contractile response. Lower intrinsic ASM tone with increased $V_{VECM}$ theoretically would reduce the likelihood that force adaptation will occur to increase contraction.

Attenuation of intrinsic ASM tone as a result of increased $V_{VECM}$ is of interest. The logical mechanism is a loss of contractile elements, or perhaps disruption of the working contractile apparatus since the absolute loss will be small. Of note, the previously observed inverse correlation between $V_{VECM}$ within the ASM layer and lung function (Jones et al., 2016b), and confirmed in this thesis (Chapter 2), was not accompanied by statistical decreases in $V_{VASM}$, albeit trends were apparent in the data (Chapter 2) and COPD subjects in Chapter 4 had reduced $V_{VASM}$. One interpretation of the data is that increased $V_{VECM}$ within the ASM layer reduces rigidity produced by contractile activation (An et al., 2002) and promotes collapse of peripheral airways, as has been demonstrated in *ex-vivo* human airways (Tiddens et al., 1999). To provide a visualisation of this phenomenon, Figure 6.1 displays an isolated bronchus from an immature animal that was collapsed under negative transmural pressure. Subsequent contraction of the ASM layer to parasympathetic nerve stimulation produced paradoxical relaxation, attributed to contraction-induced stiffening that resisted the compressive pressure (McFawn and Mitchell, 1997b). Such studies reinforce the notion that ASM tone may be beneficial under some conditions, and we speculate that compositional changes to the ASM layer in COPD could make peripheral airways more susceptible to collapse.
Figure 6.1: Paradoxical bronchodilation to parasympathetic nerve stimulation. A) an immature porcine bronchial segment was collapsed under compressive pressure; B) after contractile activation, paradoxical bronchodilation was observed. Recordings were performed using video-endoscopy. Adapted from (McFawn and Mitchell, 1997b).

The relationship between the composition of the ASM layer and induced ASM contraction was further investigated *in vivo* after bronchial challenge in subjects with and without COPD (Chapter 4). This first preliminary study did not support a strong effect of composition of the ASM layer on airway responsiveness to methacholine, with only $V_{V_{\text{ASM}}}$ within the ASM layer inversely related with induced-bronchoconstriction. These findings are in discordance with Chapter 3 where induced ASM stress (to acetylcholine or histamine) was not affected by the composition of the ASM layer. The discrepancy may be related to the different cohorts, since a higher proportion of subjects undergoing bronchial challenge were classified as having COPD. Moreover, airway narrowing capacity *in vivo* is influenced by factors other than ASM stress, including airway-parenchymal interactions (Macklem, 1991) and geometry (Lambert *et al.*, 1993). Notably, *in vitro* contractile response is normalised to ASM cross-sectional area (*i.e.*, stress), which allows the independent effect of the composition of the ASM layer to be examined. For the *in vivo* study (Chapter 4), normalised wall areas (area to epithelial basement membrane perimeter) were not related to airway responsiveness, suggesting that the composition of the ASM layer is a stronger predictor of induced-bronchoconstriction. These factors aside, a reduced $V_{V_{\text{ASM}}}$ within the ASM layer seems to favour a decrease in airway responsiveness.
in COPD, more in agreement with the proposal that airway narrowing capacity is reduced in some patients with COPD (Jones et al., 2016a).

A booming area of research over the past decade is the importance of the dynamic environment of the lung to human health and its disruption in obstructive disease (An et al., 2007). The ASM layer is stretched cyclically over the course of a 4 s tidal breath and intermittently with larger deep inspirations (DI) that occur every 6 min (Bendixen et al., 1964). Our laboratory has been intensely focused on demonstrating that bronchodilatory responses to breathing manoeuvres are elicited by direct stretch of the airway wall, initially utilising airways from animals (LaPrad et al., 2008, Noble et al., 2007, Khangure et al., 2004, Noble et al., 2004) before confirming findings on human tissue (Noble et al., 2011, Noble et al., 2013). Our model controls pressure swings across the airway (transmural) to simulate peri-bronchial pressures transmitted via parenchymal attachments. Broad findings show bronchodilation to breathing manoeuvres at the level of the airway wall, the magnitude of which is reduced by wall stiffness (Noble et al., 2007). A direct response of the ASM layer to stretch is favoured, potentially due to cross-bridge detachment (Fredberg et al., 1997) and / or the plasticity of the contractile apparatus (Seow, 2005). In subjects with COPD, there is a loss of the bronchodilatory response to DI (Scichilone et al., 2004, Scichilone et al., 2008), which is at least partially attributed to airway-parenchymal uncoupling as a result of emphysema. In Chapters 2 and 5, we examined whether the above disruption could be due to intrinsic abnormalities of the airway wall and / or stiffening of the airway as a result of hyperinflation.

Assuming that bronchodilation to breathing manoeuvres is due to an intrinsic response of the airway wall and not due to external influences such as central neural control (Schweitzer et al., 2011), an abnormal response to an applied stretch could explain reduced bronchodilation to DI. There is some evidence to support such a mechanism in asthma (Noble, 2013) in view of data from subjects in vivo (Hulme et al., 2013, Brown et al., 2001) and isolated ASM studied in vitro (Chin et al., 2012). Data
generated in Chapter 2 on the other hand provides no evidence that the airway wall responds differently to mechanical stretch, and bronchodilation to simulated DI in vitro was comparable between groups for all protocols. Reduced transmission of pressure to the airway wall during DI therefore seems more likely to impact bronchodilation to DI, although as stated above, airway stiffness is another influential variable.

The role of airway wall stiffness in determining the response to DI in COPD was examined by first comparing the specific compliance of bronchial segments from subjects with and without fixed airflow limitation (Chapter 2). Compliance, the inverse of airway stiffness (or more specifically elastance), was comparable between subject groups, under relaxed conditions, or in the presence of induced contractile activation, where compliance was reduced and/or stiffening occurred. Given that there were no changes in gross wall thickness between groups, this finding is not unexpected. Airway wall thickness is associated with reduced specific compliance of isolated human airways in vitro, and is further reduced by ASM tone (Tiddens et al., 1999).

The second aspect of the project examining the impact of airway stiffness in determining the bronchodilatory response to DI in COPD was based on our hypothesis that hyperinflation stiffens the wall in COPD. Fundamentally, even if the material mechanical properties of the airway wall are not affected by the disease process, the non-linear pressure-volume relationship of the airway results in stiffening of the airway wall in patients where dynamic hyperinflation is present. A clear change was apparent in our in vitro simulation of hyperinflation where bronchodilation to simulating breathing was substantially reduced, suggesting that hyperinflation-induced airway stiffening should be considered as a mechanism for loss of bronchodilatory response in COPD.

Findings from the above experiment are presented in Chapter 5, which is the first chapter that has preceded to publication (Cairncross et al., 2018), and was encouragingly editorialised by Professor
Jason T. H. Bates, Vermont, USA (Bates, 2018). The editorial points to an assumption of the reductionist approach where single components of the respiratory system are isolated to better understand the integrated system. Professor Bates states in the editorial “In terms of length scale, an entire living lung is not merely a scaled-up version of a single airway. In addition to critical biological factors such as tissue remodelling and mucus production, the living lung is a complex collection of many airways that often vary widely in their properties and behaviour, making its overall behaviour potentially very different to that of a single tube surrounded by ASM”. We are in agreement with these facts and indeed the hyperinflation-induced stiffening is unlikely to be universally distributed, with some airways potentially retaining their bronchodilator response and others blunted altogether. Such a scenario would contribute to ventilation inhomogeneities in COPD (van Geffen et al., 2018) and is equally relevant to diseases such as asthma where variability in airway remodelling is demonstrated between and within patients (Elliot et al., 2015, Pascoe et al., 2017) contributing to heterogeneous bronchoconstrictor patterns (King et al., 2004, Dame Carroll et al., 2015). The effect of emphysema and loss of elastic recoil should also not be disregarded which will offset the effects of hyperinflation. Ultimately, these opposing forces may contribute to a more diverse range of patient phenotypes.

**Figure 6.2: The evolving paradigm of COPD.** Red text denotes contributions from the present thesis.
A summary of how the results of the thesis contribute to our understanding of COPD pathophysiology is outlined in Figure 6.2. Increased narrowing capacity and proposed airway collapse both contribute to airflow limitation, in addition to loss of bronchodilatory response to DI, partly mediated by lung hyperinflation. Airway collapse itself could further enhance hyperinflation and cause gas trapping. Some conflicting observations are however observed. Based on findings from Chapter 4, reduced $V_{VASM}$ within the ASM layer would favour reduced AHR. Such discrepancies should be further investigated, and may be related to size of the airway and the aforementioned integrated response of the intact respiratory system.

With all this in mind, a series of intuitive experiments need to be performed. Knowledge on what ECM proteins are actually altered within the ASM layer would facilitate more informed hypotheses regarding function. Depending on the composition of the ECM, the forces transmitted may be different as has been demonstrated by An et al. (An et al., 2009). Planned analyses include collagen types I, III and V, biglycan, decorin, fibronectin; and versican. Somewhat related is whether changes in ECM components are associated with alterations in integrin expression. All these analyses will be performed in a larger number of airways from a larger number of subjects to account for variability between and within subjects. Greater phenotyping of patients is also necessary so that changes in lung function can be portioned into airway opposed to parenchymal disease, which can be achieved by computed tomographic-assessment of emphysema.

There is an increasing need to find better ways to study the ASM layer. Polarisation-sensitive optical coherence tomography (OCT) provides a fantastic opportunity to measure ASM in situ i.e., within the patient. Polarisation-sensitive OCT (Villiger et al., 2016) is sensitive to the birefringence of tissue. Birefringence is related to the aspect ratio and orientation of the structural units of tissue from the molecular to optical wavelength scale (few micrometres). Within the airway wall, the ASM forms bands of elongated cells which are orientated around the airway at roughly 90° to the long axis of the
airway (Ijpmä et al., 2017). These bands are surrounded by blood vessels, matrix and resident cells which have a much more random orientation. Therefore, it is possible to delineate the bands of ASM muscle using the birefringence of light (Adams et al., 2016). One airway collected as part of the recruitment protocol outlined in this thesis was scanned by OCT and is shown in Figure 6.3 with the ASM layer and cartilage layers outlined. Mechanical properties are also assessable through use of OCT. Changes in tissue thickness in response to applied mechanical stimuli are measured by OCT, allowing calculation of elastance. The technology is known as OCT-based elastography (Kennedy et al., 2015) and provides an opportunity to further examine whether there are changes in airway stiffness in COPD.

**Figure 6.3: Human bronchial tissue scanned by optical coherence tomography (OCT). A) scanned by OCT; and B) histological comparison. The muscle appears as blue (yellow star) and the cartilage (green star).**

In concluding, and in view of the above discussion, it is appropriate to speculate on the treatment of patients with COPD. There is a need to drastically improve treatment plans for COPD patients since current pharmacological approaches produce only a modest improvement in lung function, quality of life and exacerbation rates (Tashkin et al., 2008, Calverley et al., 2003). New approaches need to consider the differences between asthma and COPD (Jones et al., 2016a). For example, while both diseases may exhibit AHR and loss of bronchodilatory response to DI, the underlying mechanisms are different and consequently so are effective disease targets. Therapies must consider large as well as small airways, and in this regard bronchial thermoplasty is a nice demonstration of how treating a
small number of large airways can improve patient outcomes (Donovan et al., 2018). If ASM tone is beneficial in preventing peripheral airway collapse, preferential treatment of small airways could be counterproductive. Finally, once a thorough examination on how changes in composition of the ASM layer in COPD impact function has been performed, treatment strategies can be developed, which could begin with the administration of drugs targeting the components within the ASM layer, such as the integrins (Wright et al., 2014).
References

Acam 2011. Asthma in Australia.


Appendix A: Respiratory questionnaires

Respiratory symptom questionnaire

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<th>PARTICIPANT DETAILS</th>
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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? [ ] [ ]

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<th>SECTION I: RESPIRATORY SYMPTOMS</th>
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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 ear surgery

If YES, specify, including years

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________
SECTION III: SMOKING HISTORY

1 Have you ever smoked regularly as many as 7 cigs./wk (1cigar/wk or 1 oz 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>
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<tr>
<td>iii</td>
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<td>iv</td>
<td></td>
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<tr>
<td>v</td>
<td></td>
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</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>
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<tr>
<td>ii</td>
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<td>vii</td>
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<tr>
<td>viii</td>
<td></td>
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</tr>
</tbody>
</table>
If treatment is currently taken for any conditions, include in General Respiratory Questionnaire, Other Medications

<table>
<thead>
<tr>
<th>System</th>
<th>Underlying condition / surgical procedure</th>
<th>Date diagnosed dd-mm-yyyy</th>
<th>Ongoing Y N</th>
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<tbody>
<tr>
<td>Eye</td>
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<td>Sleep</td>
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<tr>
<td>Cardiovascular</td>
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<tr>
<td>Neurology</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal / reflux / indigestion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatobiliary/pancreas</td>
<td></td>
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<tr>
<td>Urology</td>
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<tr>
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<tr>
<td>Musculoskeletal</td>
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<td></td>
</tr>
<tr>
<td>Blood and Lymphatic</td>
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<tr>
<td>Stroke</td>
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<tr>
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<tr>
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<td>Thyroid</td>
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Appendix B: Participant information checklist

Participant Number: _______________________

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<th>Comments</th>
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<tr>
<td>Consent Form</td>
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<td>Consent to disclose Medical Information</td>
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<td>General Respiratory Questionnaire</td>
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<td>Asthma Control Questionnaire</td>
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<td>St George’s Respiratory Questionnaire</td>
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<td>Prick Skin Test</td>
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<td>Current Spirometry Results</td>
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<td>Previous Lung Function Results</td>
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<td>Radiography Results</td>
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**Specimen Check List**

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<th>Comments</th>
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<tbody>
<tr>
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<tr>
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<tr>
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Appendix C: Staining protocols

Haematoxylin and Eosin (4 µm sections)

Procedure:

- Bring through 3 changes of xylene - 2 min in each solution
- Bring through three changes of 100% ethanol – 2 min in each solution
- 90% ethanol – 2 min
- 70% ethanol – 2 min
- Rinse in distilled water
- Harris haematoxylin – 4 min
- Blue in running tap water – 5 min
- Eosin – 2 min
- 70% ethanol – 2 min
- 90% ethanol – 2 min
- Bring through 3 changes of ethanol - 2 min in each solution
- Bring through 3 changes of xylene - 2 min in each solution
- Dry and coverslip from xylene

Masson’s trichrome (0.5 µm sections)

- Bring through 3 changes of xylene - 2 min in each solution
- Bring through three changes of 100% ethanol – 2 min in each solution
- 90% ethanol – 2 min
- 70% ethanol – 2 min
- Rinse in distilled water
- Celestein Blue - 5 min
- Rinse in water – 2 min
- Harris haematoxylin – 5 min
- Rinse in water – 2 min
- Rinse in distilled water
- Ponceau-Fuchsin (working solution) – 10 min
- Rinse in water
- Differentiate in phosphomolybdic (until collagen is clear) - 3 min
• Rinse in distilled water
• Aniline blue – 1 min
• Rinse in acetic acid – 1 min
• Bring through 3 changes of ethanol - 2 min in each solution
• Bring through 3 changes of xylene - 2 min in each solution
• Dry and coverslip from xylene