COMPLEX LUNG PHYSIOLOGY AND AIRWAY INFLAMMATION IN ADULTS WITH ASTHMA AND FIXED AIRFLOW OBSTRUCTION

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Table of Contents

STATEMENT OF AUTHORSHIP AND ORIGINALITY I
DEDICATION II
ACKNOWLEDGEMENTS III
AUTHORSHIP ATTRIBUTION STATEMENT V
PUBLICATIONS, ABSTRACTS AND AWARDS ARISING FROM THIS THESIS VII
PUBLISHED MANUSCRIPTS VII
ACCEPTED MANUSCRIPTS VII
MANUSCRIPTS UNDER REVIEW VII
ABSTRACTS VIII
AWARDS IX
LIST OF ABBREVIATIONS XI

ABSTRACT 1

1 INTRODUCTION AND REVIEW OF LITERATURE 3

1.1. GENERAL INTRODUCTION 4
1.2. ASTHMA 5
1.2.1. DEFINITION 5
1.2.2. EPIDEMIOLOGY 6
1.2.3. RISK FACTORS 6
1.2.4. DIAGNOSIS 7
1.2.5. PHENOTYPES 9
1.2.6. MANAGEMENT 9
1.3. ASTHMA IN OLDER PEOPLE 11
1.4. NORMAL AGEING OF THE LUNG 14
1.4.1. CHEST WALL 14
1.4.2. RESPIRATORY MUSCLES 14
1.4.3. AIRWAYS 15
1.4.4. LUNG TISSUE 15
1.5. ASTHMA PATHOPHYSIOLOGY: STRUCTURAL CHANGES 19
1.5.1. AIRWAY REMODELING 19
1.5.2. LUNG TISSUE CHANGES 23
1.6. ASTHMA PATHOPHYSIOLOGY: INFLAMMATION 29
1.6.1. MEASUREMENT OF AIRWAY AND LUNG TISSUE INFLAMMATION 30
1.6.2. AIRWAY INFLAMMATION 35
1.6.3. LUNG TISSUE INFLAMMATION 38
1.7. ASTHMA PATHOPHYSIOLOGY: FUNCTIONAL CHANGES 44
1.7.1. AIRFLOW OBSTRUCTION, AHR AND HYPERINFLATION 44
1.7.2. SMALL AIRWAYS 45
1.7.3. SMALL AIRWAY DYSFUNCTION 48
1.7.4. VENTILATION HETEROGENEITY 49
1.7.5. VENTILATION HETEROGENEITY IN ASTHMA 57
1.7.6. RESPIRATORY IMPEDANCE 58
1.7.7. RESISTANCE AND REACTANCE IN ASTHMA 64
1.7.8. LUNG TISSUE MECHANICS – ELASTIC RECOIL AND COMPLIANCE 66
1.7.9. LOSS OF LUNG ELASTIC RECOIL 73
1.8. FIXED AIRFLOW OBSTRUCTION IN ASTHMA 77
1.8.1. DEFINITION AND PREVALENCE OF FAO 78
4.2.1 SUBJECTS 193
4.2.2 STUDY DESIGN 194
4.2.3 LUNG FUNCTION TESTS 195
4.2.4 STATISTICAL ANALYSES 196
4.3 RESULTS 197
4.3.1 SUBJECTS AND LUNG FUNCTION DATA 197
4.3.2 UNIVARIATE CORRELATIONS WITH SPIROMETRIC RATIO 203
4.3.3 UNIVARIATE CORRELATIONS WITH FORCED OSCILLATORY IMPEDANCE 206
4.3.4 MULTIVARIATE CORRELATIONS WITH SPIROMETRY AND FORCED OSCILLATION IMPEDANCE 207
4.4 DISCUSSION 208

References 213

5 STEROID INSENSITIVE FIXED AIRFLOW OBSTRUCTION IS NOT RELATED TO AIRWAY INFLAMMATION IN OLDER NON-SMOKERS WITH ASTHMA. 218
5.1 INTRODUCTION 219
5.2 METHODS 219
5.3 RESULTS 221
5.4 DISCUSSION 225
5.5 CONCLUSION 226
References 227

6 CONCLUSION 230
6.1 FUNCTIONAL RESIDUAL CAPACITY AND VENTILATION HETEROGENEITY MEASUREMENTS BETWEEN COMMERCIAL MBNW DEVICES ARE NOT COMPARABLE. 231
6.2 LOSS OF LUNG ELASTIC RECOIL IS ASSOCIATED WITH SMALL AIRWAY DYSFUNCTION IN THE DIFFUSION-DEPENDENT AIRWAYS (SACIN) AND AIRFLOW OBSTRUCTION (FEV1/FVC). 232
6.3 THE AGEING PROCESS CONTRIBUTES TO FIXED AIRFLOW OBSTRUCTION. 233
6.4 STEREOID INSENSITIVE FIXED AIRFLOW OBSTRUCTION IS NOT RELATED TO AIRWAY INFLAMMATION IN OLDER NON-SMOKES WITH ASTHMA. 234
6.5 FUTURE DIRECTIONS 235
6.5.1 MBNW AND MEASUREMENT OF SMALL AIRWAY FUNCTION 235
6.5.2 EXPLORING MECHANISMS CONTRIBUTING TO THE LOSS OF LUNG ELASTIC RECOIL IN ASTHMA 236
6.6 FINAL CONCLUSIONS 238
References 240

7 APPENDIX 243
References 257
Statement of Authorship and Originality

The work presented in this thesis was carried out within the Department of Respiratory Medicine at the Royal North Shore and Concord Hospitals and at the Woolcock Institute of Medical Research under the supervision of Professor Gregory King, Dr Claude Farah and Dr Cindy Thamrin. I collected all data presented in this thesis between September 2015 and July 2017, including the publication in the appendix. Data analysis and interpretation for all chapters presented in this thesis are my own work.

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Katrina O Tonga
Dedication

THIS THESIS IS DEDICATED IN LOVING MEMORY OF MY GRANDMOTHER

HELENA ADI TEIMUMU LOLOA

WHO SADLY PASSED AWAY JUST BEFORE ITS COMPLETION.
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Authorship Attribution Statement

Chapter 2 of this thesis is published as:


I designed the study, collected and analysed the data and wrote the drafts of the manuscript.

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Katrina O Tonga
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Abstracts

• Tonga K, Farah C, Thamrin C, Tang F, Santos J, Sharma P, Oliver B, King G. Lung elastic recoil, inflammation and persistent airflow limitation in asthma. Respirology 2018; 23 (Suppl.1), TP067.

• Tonga KO, Farah CS, Thamrin C, Tang FS, Santos J, Sharma P, Oliver BG, King GG. Persistent airflow limitation, lung elastic recoil and inflammation in older non-smokers with asthma. Am J Resp Crit Care Med 2018; 197:A5836

• Tonga KO, Farah CS, Nguyen C, Thamrin C, King GG. Lung elastic recoil in older asthmatics with fixed airflow obstruction. Am J Respir Crit Care Med 2017; 195:A4859

• Tonga KO, Farah CS, Thamrin C, King GG. Lung elastic recoil in older asthmatics with fixed airflow obstruction. Concord Hospital Early Career Research Presentation 2017.


• Tonga KO, Durack T, King GG. Frequency dependence of compliance and fixed airflow obstruction in older people with asthma. Flow Volume Underworld Meeting 2017

• Tonga KO, Farah CS, Thamrin C, King GG. Blood neutrophils relate to lung volume and DLCO in older asthmatics with fixed airflow obstruction. Respirology 2017; 22 (Suppl. 2), 101-193.
• Tonga KO, Thamrin C, Farah CS, Robinson PD, King GG. In-vivo and in-vitro functional residual capacity comparisons between multiple breath nitrogen washout devices. European Respiratory Journal Sept 2016; 48 (Suppl 60), PA2222

• Tonga K, Thamrin C, Farah CS, King GG. Comparison between 2 commercial multiple breath nitrogen washout devices and an in house device. Respirology 2016; 21 (Suppl. 2), 12-20


Awards

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• 14th Annual Newcastle Asthma Meeting 2018 – Best Post-Doctoral Clinical Presentation

• Thoracic Society Australia and New Zealand (TSANZ) Ann Woolcock New Investigator Award 2018 – Finalist

• American Thoracic Society (ATS) – Assembly on Respiratory Structure & Function Abstract Scholarship 2017

• ATS – Asia Pacific Society of Respirology Travel Award 2017

• TSANZ NSW Branch Meeting 2017 – Best Oral Presentation (Airways Disease)
• Concord Hospital Early Career Research Award 2017 – Winner (Postgraduate Student Category)

• Woolcock Biennial Postgraduate Symposium 2017 – Best Oral Presentation

• Centre of Excellence in Severe Asthma Travel Grant 2017 and 2019

• Khancoban Asthma Australia Travel Grant 2017

• Concord Hospital Travel Scholarship 2017 and 2018

• TSANZ Travel Grant 2017 and 2018

• University of Sydney Postgraduate Research Support Scheme Award 2015-2017

• Australian Postgraduate Award 2014-2017
List of Abbreviations
ACO: asthma COPD
ACQ-5: asthma control questionnaire-5
ADAM33: adisintegrin and metalloprotease 33
AHR: airway hyper-responsiveness
ASM: airway smooth muscle
B/A: reflects lung elastic recoil
BAL: bronchoalveolar lavage
BMI: body mass index
CDI: convection-dependent inhomogeneity
CEV: cumulative expiratory volume
C_{FLS}: collapsibility of the flow limiting segment
COPD: chronic obstructive pulmonary disease
CT: computed tomography
CXCL: CXC motif chemokine ligand
DCDI: diffusion-convection-dependent inhomogeneity
ECM: extracellular matrix
EGFR: epidermal growth factor
EM: EcoMedics
ERS/ATS: European Respiratory Society/American Thoracic Society
FAO: fixed airflow obstruction
FEF25-75%: forced expiratory flow at 25-75% of FVC

FEV1: forced expiratory volume in 1 second

FOT: forced oscillation technique

FRC: functional residual capacity

FVC: forced vital capacity

Gus: conductance of the upstream segment

ICS: inhaled corticosteroid

IL: interleukin

ILC: innate lymphoid cells

IQR: interquartile range

K: reflects lung compliance

LABA: long-acting beta agonist

LCI: lung clearance index

MBNW: multiple breath nitrogen washout

MFSR: maximal flow static recoil

MMP: matrix metalloprotease

N2: nitrogen

ndd: new diagnostic design

P-V: pressure-volume

P_{ALV}: alveolar pressure
$P_{AO}$: pressure at the airway opening

$P_{ATM}$: atmospheric pressure

$P_{CW}$: trans-chest wall pressure

PEF: peak expiratory flow

$P_L$: trans-pulmonary pressure

$P_{PL}$: pleural pressure

$P_{RS}$: trans-respiratory system pressures

RBM: reticular basement membrane

$R_{rs}$: respiratory resistance

RV: residual volume

Sacin: ventilation heterogeneity in the diffusion-dependent airways

Scond: ventilation heterogeneity in the conductive-dependent airways

SD: standard deviation

SIII: phase III slope

TH$_1$: T-helper 1

TH$_{17}$: T-helper 17

TH$_2$: T-helper 2

TLC: total lung capacity

TSLP: thymic stromal lymphopoietin

VEGF: vascular endothelial growth factor
VH: ventilation heterogeneity

$V_{max}$: maximal expiratory flow

WIMR: Woolcock Institute of Medical Research

$X_{rs}$: respiratory reactance

$Z_{rs}$: respiratory impedance

$\alpha$-SMA: alpha-smooth muscle actin

$O_2$: oxygen
Abstract

Background:

Fixed airflow obstruction (FAO) occurs in asthma despite adequate treatment and no or minimal smoking history. FAO in asthma is more common in older people or those with long-standing disease and associated with poor outcomes. Airflow obstruction occurs in the small airways and is thought to be due to airway remodelling and driven by inflammation. Changes to the lung tissue, which may result in alteration of the lungs elastic properties such as loss of lung elastic recoil, may also contribute. The underlying mechanisms leading to FAO in asthma are poorly understood.

Aim:

To explore the physiological properties of both the airways and lung tissue and airway inflammation in older non-smokers with asthma, and to assess how they may contribute to FAO.

Method:

Non-smoking adults >40 years old with asthma were treated with two months of high dose inhaled corticosteroid/long-acting beta-agonist (ICS/LABA). Subsequently standard lung function and small airway function was measured using the forced oscillation technique (FOT) and the multiple breath nitrogen washout test (MBNW). Lung elastic recoil was measured using the oesophageal balloon technique. Airway inflammation was measured using bronchoalveolar lavage fluid obtained during bronchoscopy.
Results:

Non-smoking adults with asthma (n=19) demonstrated moderate FAO; small airway dysfunction, as measured by FOT and MBNW; increased lung compliance and loss of elastic recoil and variable airway inflammation. Worse airflow obstruction was associated with increased lung compliance. Increased airway resistance and small airway dysfunction in the acinar airways was associated with a loss of lung elastic recoil. Cross-sectional assessment of airway inflammation was not associated with lung function impairment.

Conclusion:

Changes to the lungs elastic properties results in increased compliance or reduced lung elastic recoil and make a significant contribution to FAO and small airway dysfunction in older non-smokers with asthma. ‘Lung remodelling’ is a potential pathological process leading to lung tissue changes and may be an alternate asthma paradigm. Underlying cellular mechanisms need further investigation.
Chapter 1

1 Introduction and review of literature
1.1. General Introduction

Asthma is characterized by variable airflow obstruction that occurs intermittently and usually reverses either spontaneously or with bronchodilator therapy (1). However, in older people with asthma airflow obstruction can become fixed or irreversible (2-5). Fixed airflow obstruction (FAO) can occur despite appropriate treatment (3-5) and despite no smoking history (2, 4, 5) or exposure to noxious substances (6). The consequences of FAO in asthma are detrimental and associated with poor outcomes and quality of life (4, 5, 7-10) however the underlying mechanisms are not well understood.

FAO in asthma is thought to be due to airway remodelling, structural changes to the airway wall that result in airway wall thickening (11-13). This causes subsequent narrowing of the airway lumen and airflow obstruction, which occurs in the small airways (14, 15). In addition to airway remodelling, structural changes outside the airway wall occur in asthma (16-18). That is changes in the surrounding lung tissue, a form of ‘lung remodelling’. Changes in the lung tissue can cause a loss of lung elastic recoil in asthma (19-21) and in older people (22). With loss of elastic recoil, the tethering force of the lung tissue to the airways is reduced and the force to keep airways open. Therefore alterations in lung tissue may also contribute to FAO in asthma.

Chronic airway inflammation plays an important role in asthma and is typically associated with TH₂ and eosinophilic inflammation (23-27). However there is increasing interest in the role of non-eosinophilic and neutrophilic inflammation. Increased airway neutrophils are seen with increasing age in both asthma (28-31) and healthy people (32). In addition, sputum neutrophilia
in asthma is associated with impaired lung function (33, 34) and severe and incompletely reversible airflow obstruction (10).

Prevention and treatment of FAO in non-smokers with asthma remains a challenge due many unanswered questions about this asthma phenotype. The airways, lung tissue and inflammation all play an important role with the addition of the ageing process. This thesis will examine physiological abnormalities in the small airways and lung tissue, as well as explore airway inflammation, in older non-smokers with asthma and FAO to increase our understanding about the underlying mechanism of this phenotype.

1.2. Asthma
1.2.1. Definition
Asthma is a heterogeneous disease characterized by chronic airway inflammation. It is defined by typical respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough, which often vary over time and in intensity, and the presence of variable airflow obstruction (1, 35). Airflow obstruction occurs in response to inhaled stimuli, such as cold air or allergens and usually reverses either spontaneously or with bronchodilator therapy. The site of airflow obstruction is in the small airways (14, 15), although both the small and large airways are affected (36, 37).

The current paradigm in asthma is that airflow obstruction is due to airway remodelling. Airway remodelling refers to structural changes in the composition, quantity and organization of the cellular and molecular components of the airway wall (11, 13, 38) and will be discussed in more detail later in this chapter. The interplay between airway remodelling and
complex inflammatory signalling processes results in airway hyper-responsiveness (AHR). AHR is a hallmark of asthma and can be defined as an exaggeration of airway narrowing in response to low-dose stimulation of airway smooth muscle (39).

1.2.2. Epidemiology
Asthma is one of the most common chronic diseases (40) and affects all age groups. It is estimated that 300 million people worldwide have asthma with geographical differences in asthma prevalence (40, 41). The incidence is increasing and consequently significant health problems have arisen globally (42). Individuals affected by asthma place an increasing burden on society due to the disability, high health care utilization and poor quality of life it causes (1, 43, 44).

1.2.3. Risk factors
Risk factors associated with asthma include lung function abnormalities at an early age (45) and atopic conditions like atopic dermatitis and allergic rhinitis (46-48). An increased risk of asthma may also be associated with certain occupations such as nursing and cleaning (49) and active and/or second-hand smoking (50-53), in particular maternal smoking (54). There is also a familial predisposition and genetic link associated with asthma. The ADIsintegrin And Metalloprotease 33 (ADAM33) gene (55, 56) has been implicated in asthma development and disease progression, with polymorphisms showing an association with an accelerated forced expiratory volume in 1 second (FEV₁) decline (56). Obesity is both a risk factor and disease modifier of asthma (57). Increased body mass index (BMI) is
associated with a greater risk of developing asthma (58, 59) and asthma severity (58). Respiratory infections, both viral and bacterial, are known to trigger asthma exacerbations however it is not known whether respiratory infections cause asthma or are a protective factor (60, 61). The interaction between infection and airway inflammation in asthma is complex and how infection affects individuals may be dependent on the specific type of infection, genetic susceptibility, age, presence of atopy and lung microbiome. These risk factors may be a consequence of complex interactions between genetic and environmental factors.

1.2.4. Diagnosis
The diagnosis of asthma is based on a history of typical respiratory symptoms and demonstration of variable airflow obstruction (1). Evidence of variable expiratory airflow obstruction should ideally be demonstrated early, as characteristic asthma features may improve spontaneously or with treatment. Variable expiratory airflow obstruction can be confirmed by using one of the following tests: peak expiratory flow, spirometry, bronchial challenge test or exercise challenge test (1). Diagnostic criteria for making the diagnosis of asthma are highlighted in table 1.
Table 1.1 Diagnostic criteria for asthma in adults.

<table>
<thead>
<tr>
<th>Diagnostic feature of variable expiratory airflow limitation</th>
<th>Criteria for making the diagnosis of asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Documented excessive variability in lung function in one of more of the tests below <strong>AND</strong> documented airflow limitation</td>
<td>The greater the variations, or the more occasions excess variation is seen, the more confident the diagnosis. At least once during diagnostic process when FEV₁ is low, confirm that FEV₁/FVC is reduced.</td>
</tr>
<tr>
<td>Positive bronchodilator reversibility test (more likely to be positive if short acting and long acting bronchodilators are withheld for ≥4 hours and ≥15 hours, respectively)</td>
<td>Increase in FEV₁ of &gt;12% and ≥200 mL from baseline, 10-15 minutes after 200-400 mcg albuterol or equivalent (greater confidence if increase is &gt;15% and &gt;400 mL).</td>
</tr>
<tr>
<td>Excessive variability in twice-daily PEF over 2 weeks</td>
<td>Average daily diurnal PEF variability &gt;10%</td>
</tr>
<tr>
<td>Significant increase in lung function after 4 weeks of anti-inflammatory treatment</td>
<td>Increase in FEV₁ by &gt;12% and &gt;200 mL (or PEF by &gt;20%) from baseline after 4 weeks of treatment, outside respiratory infections</td>
</tr>
<tr>
<td>Positive exercise challenge test</td>
<td>Fall in FEV₁ of &gt;10% and &gt;200 mL from baseline</td>
</tr>
<tr>
<td>Positive bronchial challenge test</td>
<td>Fall in FEV₁ from baseline of ≥20% with standard doses of methacholine or histamine, or ≥15% with standardized hyperventilation, hypertonic saline or mannitol challenge</td>
</tr>
<tr>
<td>Excessive variation in lung function between visits (less reliable)</td>
<td>Variation in FEV₁ of &gt;12% and &gt;200 mL between visits, outside of respiratory infections</td>
</tr>
</tbody>
</table>

Adapted from GINA Global Strategy for Asthma Management and Prevention 2017 (1).

FEV₁: forced expiratory volume in 1 second; PEF: peak expiratory flow (highest of three readings).
1.2.5. Phenotypes
The heterogeneous nature of asthma has lead to characterization of the disease by different phenotypes (10, 62, 63). The phenotypes are classified according to consistent groupings of demographic, clinical and pathophysiological features. However phenotypes are also evolving to include molecular and genetic features (62). Commonly recognized phenotypes include: early onset allergic asthma, non-allergic asthma, late-onset asthma, exercise induced asthma, asthma with fixed airflow obstruction and obesity related asthma (62). By phenotyping asthma, more targeted and personalized therapy can be used for specific phenotypic features. However ongoing work is needed to better identify and characterize asthma phenotypes.

1.2.6. Management
The goals of asthma management are to control symptoms and to reduce future risk of adverse outcomes (1). Pharmacological therapy will not be discussed in detail however includes inhaled short-acting beta-agonists, inhaled glucocorticosteroids with or without long-acting beta-agonists, leukotriene receptor antagonists and theophylline (1). Acute asthma exacerbations and/or difficult to control asthma may be treated with a short course of oral glucocorticosteroids (1, 64-67). Escalation in treatment is required for severe asthma and may include the addition of other therapies such as a long-acting muscarinic inhaler (68), biological agents (69-73) and bronchial thermoplasty (74-76). The patient population for whom these newer treatments are targeted needs ongoing research (74), as their role in asthma
treatment and which asthma patients will benefit the most is not yet well established.
1.3. Asthma In Older People

Asthma in the older population is common (77-82) and the prevalence is increasing, which may in part be due to people living longer. The exact prevalence of asthma in older people is difficult to determine as it is often under or misdiagnosed (80, 83, 84). Asthma prevalence in people over the age of 65 years in European and US populations is reported to be between 1.8–10.9% (85, 86). The highest rate of asthma has been reported in Australia (87), with the greatest burden in older individuals, where there is an increased prevalence of current asthma (>13% in women >75 years of age).

Multiple factors contribute to the diagnostic challenge of asthma in older people, including variable clinical presentation, disease heterogeneity and poor perception of symptoms. Cigarette smoking or a history of passive exposure can be a confounding factor as smokers may not necessarily be excluded from studies in older asthmatics (88). Diagnostic tests such as spirometry are also problematic because older people often have difficulty performing lung function tests (89, 90). There are limitations in the reference values used for currently available physiological and biomarker tests, such as exhaled nitric oxide. Predictive equations may not incorporate age at the extreme upper end (i.e. > 80 years) and older non-white ethnicities (91). Furthermore there is no clear consensus on how to modify and incorporate older age into interpretation of these tests.

Asthma in the older population is associated with poor quality of life and significantly higher medical care costs (85, 92, 93). Compared to younger people with asthma, there is also increased mortality and morbidity (92, 94-
This can be a consequence of poor medication adherence due to difficulty administering inhaler medication (98, 99), contributed to by dexterity issues (100) and cognitive impairment (98, 101). Under-treatment as a result of misdiagnosis adds to poor quality of life and may impact morbidity and mortality in this older population (81, 102). The associated increase in mortality and morbidity and misdiagnosis in older asthmatics adds to the increased burden on society.

Differences exist between asthma in younger and older people and can be attributed to various factors including the ageing process and effects of the disease itself (86, 94, 103). Structural changes (104-107), low grade inflammation (108) and immunosenesence occur as part of the ageing process. With increasing age, in both healthy people (32, 109) and in people with asthma (28, 109-111), there may be an associated increase in airway neutrophils. Eosinophil function may also be altered or reduced in older asthmatics (112).

Other factors contributing to differences between young and old people with asthma (109, 113) include longer duration of disease (109, 114), under treatment (84-86, 115) and/or treatment unresponsiveness. Diminished airway beta-adrenoreceptor responsiveness occurs in older people with asthma suggesting dysfunctional airway beta-adrenoreceptors (116). Older asthmatics can have persistent lung function abnormalities despite treatment with inhaled and/or oral corticosteroids (3, 117) suggesting steroid insensitivity or unresponsiveness.
Studies on treatment of asthma in the older population are limited therefore treatment recommendations are based on evidence from younger asthmatics (1, 86, 94). A multidisciplinary management approach is recommended due to multiple clinical issues faced by older asthmatics (118).

Although an asthma phenotype in the older population has not yet been well defined, older people with asthma can develop irreversible of FAO (5, 117). It is likely that the combined effects of asthma and the ageing process influence FAO development. Because the underlying mechanisms of asthma in older people and FAO are poorly understood, treatment remains a challenge. Potential mechanisms contributing to FAO in older people with asthma will be explored in this thesis. Before discussing the underlying pathophysiology in asthma, the structural changes that occur with normal ageing will be reviewed.
1.4. Normal Ageing of the Lung
Lung function progressively declines with age, more so when associated with disease (119-121) and may be due to age-related structural changes to the respiratory system. These structural changes can be variable between and within subjects (86, 122) and can result in a decrease in chest wall compliance, reduction in respiratory muscle strength, loss of lung elastic recoil and airflow limitation (104). Adequate lung function and gas exchange can however be maintained by the ageing respiratory system, in the absence of disease (123).

1.4.1. Chest wall
The chest wall can change with age because the rib cage and its articulations (costal-cartilage and rib-vertebral) can undergo calcification with age. The intervertebral disc spaces can also narrow (104) resulting in a more rigid and stiff rib cage (104, 124). Kyphosis and an increase in the antero-posterior diameter can also change the shape of the chest wall (124, 125). A rounder thorax (‘barrel chest’) is formed and may be related to partial and/or complete vertebral fractures, as a result of age-related osteoporosis (126). Alterations to the chest wall consequently modify chest wall compliance, which decreases with age (127-130). Changes in the chest wall may also alter the shape of the underlying lung (131).

1.4.2. Respiratory muscles
The main muscle of respiration is the diaphragm and changes in its shape can impair the force it generates (132-134). The shape of the diaphragm can change as a consequence of age-associated changes to the rib cage (125),
reduction in chest wall compliance and increase in functional residual capacity (104, 128). Elderly people have significantly reduced diaphragmatic strength compared to younger people (132) and respiratory muscle strength may also be reduced due to deficient nutritional status (135), which is commonly seen in the elderly. Skeletal muscle undergoes age-related changes including a decrease in muscle mass, decrease in the number of muscle fibres as well as a loss of peripheral motor neurons (134, 136). This results in reduced skeletal muscle strength, which may also affect the respiratory muscles. Respiratory muscle function may be affected by other common co-morbidities in older people such as cerebrovascular disease and chronic heart failure (137).

1.4.3. Airways
Airways from older healthy lungs differ morphologically from those in young people (138). The airway walls in older lungs can be irregular and the airways may not taper off or may be blocked with square or rounded ends. These changes are thought to be due to mucus impaction or inflammatory changes in the airway wall. The airway changes seen in older lungs are also seen in people with obstructive airways disease but are more common and prominent in diseased lungs. Airway stiffness in older healthy lungs is increased compared to young healthy lungs, which suggests airway remodeling may also increase with age (138).

1.4.4. Lung tissue
The lung tissue is made up of a network of fibres including collagen, elastin and proteoglycans. The total lung content of collagen does not change with age (139), however collagen fibres become more stable and more resistant to
denaturation (140) due to increased numbers of inter-molecular links. Reported changes to elastin that occur with age are conflicting (139) as elastin content may increase (141). In contrast, elastic fibres in the respiratory bronchioles and alveoli can degenerate after the age of 50 years with the fibres appearing ruptured and coiled (105). These changes are prominent around the alveolar ducts therefore the alveolar ducts dilate, the alveoli enlarge and become wider and more shallow (104, 105). With age, the presence of pseudo-elastin can also cause changes to the spatial arrangement and cross-linking of the elastic fibres (142).

The inter-alveolar wall distance increases with age (107, 143) and the surface-volume ratio decreases therefore surface area also decreases. This is thought to occur from the third decade of life onwards. Flattening of the internal surface of the alveoli may also occur and result in reduction in the alveolar surface (105). Airspace enlargement with age occurs in a relatively homogenous pattern, as opposed to being distributed heterogeneously as seen in emphysema due to smoking (105) (figure 1.1). Additionally, in contrast to smoking-related emphysema there is no alveolar destruction (105). Animal studies in senescence-accelerated mice have also shown similar changes of relatively homogeneous enlargement of alveolar duct size with age (144). Alveolar wall destruction was not demonstrated in these mice and cellular inflammation was not prominent, suggesting these changes were not due to inflammation, like that seen in emphysema (144).

The ageing lung has been termed ‘senile emphysema’ (105, 145) because of the similarities in physiological functional changes with smoking-related
emphysema. Age-related changes to the lung tissue are thought to account for the reduction in lung elastic recoil pressure that occurs with age (22). Therefore structural airway and lung tissue changes that occur with ageing could accentuate functional abnormalities seen in older people with asthma. The next sections will review the structural and functional changes than can occur in asthma.
Figure 1.1

From Verbeken et al (105).

Example of normal lung (top panel) and ‘senile emphysema’ (bottom panel).

The bottom panel demonstrates homogenous enlargement of the respiratory airspaces.
1.5. Asthma Pathophysiology: Structural Changes

1.5.1. Airway remodeling

The human airway is made up of different layers including the epithelium with an underlying sub-epithelial basement membrane, a sub-mucosal layer, mucous glands and extra-cellular matrix (ECM) comprised of fibroblasts embedded in connective tissue and airway smooth muscle (ASM). In asthma, the airways can undergo structural changes, termed airway remodelling (11-13). Airway remodelling encompasses epithelial abnormalities (146-148), changes to the mucus-secreting structures (149-151) and ECM (152-155), increased ASM mass (156, 157) and increased vascularity (158) (figure 1.2). Airway remodelling can affect both the small and large airways (13, 36, 159, 160) and the components of the airway wall that are affected varies depending on the size of the airway involved (36). Inflammatory changes occur in both the small and large airways either as a result of airway remodelling or a consequence. The inflammatory changes are either more significant or found predominantly in the small airways (11, 36, 37, 157, 159-161). However studies also show there is also more inflammation in the larger airways (162). Nevertheless, airway remodelling causes airway wall thickening, which can occur in both the inner and outer airway wall (12, 13). These changes may be transient or permanent and subsequently result in airway lumen narrowing. Airway remodelling has been confirmed in multiple patho-histological studies (36, 149, 163) and radiologically (164, 165).
Figure 1.2

From Bai et al (13).

A cartilaginous airway in a subject with fatal asthma showing structural airway remodelling changes.
A common finding in asthma is damage to airway epithelium (146-148), a change that is seen in mild (146) and newly diagnosed asthma, suggesting mucosal inflammation occurs early in the disease (166). Shedding of the epithelium can also occur at a higher rate in people with asthma compared to healthy people (167-169).

Changes to the mucus-secreting structures include mucus gland hypertrophy (150, 151) and goblet cell hyperplasia resulting in mucus hyper-secretion (149, 170, 171). Gene expression profiles may contribute to increased mucus production in asthma. MUC5AC, a mucus expression gene is increased in people with asthma compared to healthy controls (171). Elevated production of CCL20 by ASM cells, possibly due to dysregulated expression of the anti-inflammatory miR-146a-5p is also associated with increased levels of chronic mucus hyper-secretion in asthma (172).

The ECM is made up of a network of inextensible fibres (collagen), elastic fibres (elastin) and other proteoglycans. Sub-epithelial fibrosis (155), sub-epithelial thickening, increased numbers of activated fibroblasts and myofibroblasts (154) and increased collagen constitute some of the ECM changes in asthmatic airways (152, 153, 173). Sub-epithelial fibrosis may include increased levels of collagens 1, 3 and 5 in the sub-epithelial layer and sub-mucosal layer (13, 155, 174). These changes can contribute to increased ECM deposition and therefore airway remodelling.

Elastic fibre structure and content differs between asthma and healthy controls (175, 176) and can vary between small and large airways (176). Elastic fibre changes in asthmatic airways include fragmentation; altered
structure (175, 176) or the elastic fibres may disappear altogether (175). In contrast to collagen, elastic fibres may be reduced in the immediate sub-epithelial layer (17, 176) and increased in the sub-mucosal layer (176, 177). Conflicting data suggests elastic fibre content or the proportion of elastic fibres is no different between airways from asthmatics and healthy controls, (178). The proportion of elastic fibres is also no different between mild and moderate-severe asthma, even after inhaled and oral glucocorticosteroid treatment (178).

Increased smooth muscle mass occurs as a result of ASM cell hyperplasia and/or hypertrophy (12, 157, 179). Variable ASM changes such as hyperplasia (180) occur predominantly in the central airways (157). In contrast, there may be no ASM hyperplasia but ASM hypertrophy in both the small and large airways, with little involvement of the central airways (13). Nevertheless, changes in the ASM can cause airway wall thickening and luminal narrowing, which can be further exacerbated by ASM contraction (37). ASM cell changes cause an increase in ASM thickness, which is associated with increased maximal airway narrowing when measured in vitro in bronchial segments (181).

Vascular changes in asthma include angiogenesis and an increase in vessel size and number (12, 182). However findings are inconsistent because histological studies have evaluated bronchial circulation in the large airways and pulmonary circulation in the peripheral airways (12, 13, 182). Vascular changes may be due to an imbalance between primary angiogenic growth factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1
VEGF can increase abnormal blood vessel permeability (184), which leads to vessel dilation and oedema, further contributing to airway wall thickening. Recent quantitative CT analyses has suggested pulmonary vascular pruning or loss of peripheral pulmonary vasculature occurs in asthma (185), a phenomenon usually associated with chronic obstructive pulmonary disease (COPD). More vascular pruning was also found to be associated with asthma severity, control and an increased risk of asthma exacerbations (185). In contrast, another study in fatal asthma showed that pulmonary arteries adjacent to the peripheral airways showed no structural differences compared to healthy controls. However there was more eosinophilic inflammation in the pulmonary artery adventitial layer in fatal asthma, suggesting asthma may also be a disease of the pulmonary vasculature and not just the airways (161).

1.5.2. Lung tissue changes

The lung tissue is made up of an enormous number thin-walled alveoli lined by epithelium, alveolar septa comprised of interstitial cells and ECM (collagen, elastin, proteoglycans) and a thin liquid film cover, pulmonary surfactant (186). Changes in the lung tissue such as emphysema (187) are typically associated with smoking and COPD, however can also occur in asthma (16, 18). The evidence in asthma is limited though substantiated in post-mortem studies with small numbers. Importantly, the lungs examined in these studies were from non-smoking adults (16-18).

Emphysematous changes in non-smokers with asthma have been demonstrated in post-mortem lungs from older people (>40 years old) (16, 18). The emphysematous changes were trivial on macroscopic examination.
and when lungs were assessed using high-resolution computer tomography imaging (16, 18). However on histological examination, there was destruction of the acinar airspace walls (16, 18), consistent with emphysema (figure 1.3). Microscopically, mild diffuse centri-lobular emphysema was observed predominantly in both upper lobes and the middle lobe. Within these same lungs areas of normal tissue was also demonstrated, suggesting a heterogeneous distribution of lung tissue changes. Intra-alveolar chord diameter (Lm) was within normal limits (<300 mm) in areas of normal lung tissue and increased in emphysematous regions (16, 18). In addition, areas of ‘senile lung’ such as alveolar duct ectasia, near homogenous alveolar hyperinflation and absence of tissue breakdown were also shown, consistent with age-related changes (145). Typical changes of airway remodelling were also seen in these post-mortem lungs, in keeping with the diagnosis of asthma (16, 18). Together these findings would support the distribution of tissue changes being heterogeneous, similar to what is thought to occur in the airways (i.e. heterogeneous airway remodelling) (36).
Figure 1.3

From Gelb et al (16).

Microscopic section from the right upper lobe of a 72 year-old non-smoking female with lifelong asthma. This demonstrates mild centri-lobular emphysema with irregularly enlarged air spaces and fractured alveolar septa (arrows). TB: terminal bronchioles, BV: blood vessel.
Other structural changes observed in the lung tissue in asthma include an increase in ECM fibres (188). This may involve an increase in the amount of myofibroblasts (188, 189), a type of fibroblast thought to be involved in tissue repair (190). A marker of myofibroblasts, alpha-smooth muscle actin (α-SMA), is increased in lung tissue cells, alveolar walls and alveolar ducts in asthmatics (both fatal and non-fatal) compared to healthy controls (189). The increase in α-SMA cells is thought to either be due to an adaptive response to biomechanical airway abnormalities or due to genetic expression (189). Alveolar interstitial myofibroblasts are also increased in uncontrolled asthmatics compared to both well-controlled asthmatics and non-asthmatics (188).

The lung tissue is connected to the outer wall of the small airways via alveolar attachments. Disruption or abnormalities of alveolar attachments have been demonstrated on histological examination of post-mortem lung from non-smoking adults with fatal asthma (17) (figure 1.4). Compared to healthy controls without a diagnosis of lung disease, the number and proportion of abnormal alveolar attachments in asthmatic lungs were increased (17). Interestingly, emphysema was not demonstrated in these asthmatic lungs however adequate pathological assessment of lung specimens may have been limited by experimental techniques (17).
A and C: Small airway of a control subject. Alveolar septa attach directly to the outer layer of the airway (small arrows). Stained with haematoxylin and eosin.

B and D: Small airway of a subject with fatal asthma. Mucus plugging, goblet cell metaplasia, thickened airway smooth muscle layer (ASM) and peribronchial inflammation are demonstrated. The arrowheads indicate abnormal or ruptured alveolar attachments. Stained with haematoxylin and eosin.

E: Elastic fibre staining in the outer wall (blue line) and peri-bronchial septa (asterisk) of a control subject.

F: Elastic fibre staining in the outer wall (blue line) and peri-bronchial septa (asterisk) of a patient with fatal asthma. Elastic fibre content is decreased compared with (E). Weigert staining.
ECM and elastic fibre content in the outer airway walls is also altered in asthma (17) and may therefore contribute to abnormal alveolar attachments. Outer airway wall ECM components such as collagen 1 and fibronectin are increased in asthma (191). Whereas elastic fibre content in the outer walls of the small airways and in the peri-bronchiolar septa (i.e. site of alveolar attachments) is decreased in asthma compared to healthy controls (17). Elastic fibre content in the peri-bronchial septa is correlated with the number of normal alveolar attachments in both asthma and healthy controls, suggesting elastic fibres may contribute to alveolar detachment. In contrast, in distal alveoli elastic fibre content was no different between fatal asthmatics and healthy controls (17). Similarly, no difference in the relative proportions of elastic fibre in lung tissue and alveolar/bronchial attachments zones were seen in lungs from fatal asthmatics when compared to non-asthmatic sudden death controls (178).

The airways and lung tissue are a complicated structure and structural changes that occur in asthma are heterogeneous, in keeping with the nature of the disease. Changes in the distal lung, including the outer airway wall/adventitia, distal airways and alveoli likely contribute to alteration in airway-lung inter-dependence and airway mechanics. There are many intertwined and complex pathophysiological mechanisms that contribute to asthma however are poorly understood. Proposed mechanisms and the subsequent functional abnormalities will be discussed in the next sections.
1.6. Asthma Pathophysiology: Inflammation

Inflammation is thought to be the main driving factor of airway remodelling and lung tissue changes in asthma. However, it is not clear whether inflammation is causative or complimentary to these changes and whether the same inflammatory processes occur in both the airways and lung tissue. Nonetheless, complex interactions between inflammatory cells from both the innate and adaptive immune system, genetic and environmental factors contribute to asthma pathogenesis (192-194).

Inflammation involves expression and release of many cells and complex signalling pathways that are mediated by different inflammatory proteins such as cytokines, chemokines, growth factors, inflammatory enzymes and receptors (24). The inflammatory process may start with airway epithelial injury resulting in a defective bronchial-epithelial barrier therefore allowing microorganisms, environmental allergens and toxins to enter and initiate an inflammatory cascade (12, 195, 196). Damage to the epithelium itself from environmental or mechanical stress can also stimulate the release of inflammatory mediators, which further drives the inflammatory process (12, 148). Epithelial damage and the inflammatory cascade may be associated with an impaired repair process, disruption of normal cell development (148) and increased proliferation of both normal and abnormal ECM proteins such as fibroblasts and myofibroblasts (148, 154).

Inflammation in the airways may also activate a pro-inflammatory process that can cause changes in the lung tissue. A proteolytic cascade has been
proposed as an underlying mechanism leading to subsequent lung tissue destruction (16, 18, 197, 198).

1.6.1. Measurement of airway and lung tissue inflammation
Various techniques have been used to measure airway inflammation using sputum (199-201) and/or bronchoalveolar lavage (BAL) and endobronchial biopsies obtained during bronchoscopy (202-206). Lung tissue samples can also be obtained by transbronchial biopsy however this can be challenging because of the invasive nature of the technique (207-209). Therefore lung tissue from asthmatic lungs is often obtained post-mortem (16, 18, 160, 191, 210). Less invasive measures have been used as a surrogate marker inflammation, such as exhaled nitric oxide and peripheral blood eosinophils, as a measure of eosinophilic inflammation (211).

Airway inflammation in asthma is heterogeneous, with granulocytes such as eosinophils and neutrophils, contributing to the inflammatory process. The amount and type of granulocytic cells in sputum has been used to identify four distinct inflammatory asthma phenotypes (201). These phenotypes have been classified according to the predominant inflammatory cell type present, namely eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic asthma (201, 212) (table 2). Although there is no standardized definition, a ≥2-3% cut off for eosinophils and ≥61% or 65.3% for neutrophils in sputum is often used to define sputum eosinophilia and neutrophilia, respectively (62, 212-214). The cut off points used to determine the amount of inflammatory cells present can vary between studies depending on the type of sample used
(i.e. sputum versus BAL and biopsy) (215). References ranges also need adjustment with age as neutrophils can increase with age (216).

Table 1.2 Inflammatory phenotypes in asthma.

<table>
<thead>
<tr>
<th>Inflammatory phenotype</th>
<th>Induced sputum cut-points</th>
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</thead>
<tbody>
<tr>
<td>Neutrophilic asthma</td>
<td>Neutrophils &gt;61%</td>
</tr>
<tr>
<td>Eosinophilic asthma</td>
<td>Eosinophils &gt;3%</td>
</tr>
<tr>
<td>Mixed granulocytic asthma</td>
<td>Eosinophils &gt;3%</td>
</tr>
<tr>
<td></td>
<td>Neutrophils &gt;61%</td>
</tr>
<tr>
<td>Paucigranulocytic asthma</td>
<td>Eosinophils &lt;3%</td>
</tr>
<tr>
<td></td>
<td>Neutrophils &lt;61%</td>
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Adapted from Gibson et al (212).

Four distinct inflammatory phenotypes can be identified based on the amount of eosinophils and/or neutrophils present.
Asthma is typically associated with eosinophilic and TH2 driven inflammatory pathways (23, 194, 217). However non-eosinophilic and neutrophilic inflammation can occur and may be driven by TH1 and TH17 inflammatory pathways (218-220). These pathways consist of distinct immune responses, which are regulated by sub-populations of CD4+ T cells known as T helper 2 (TH2), T helper 1 (TH1) and T helper 17 cells (TH17), respectively (24) (figures 1.5 and 1.6).
Figure 1.5

From Papi et al (221).

Mechanisms and characteristic pathological features of asthma immunopathology.
Figure 1.6
From Lambrecht et al (193).

Relative roles of T-helper 2 (TH2) and innate lymphoid cells (ILC) in two forms of eosinophilic asthma.
1.6.2. Airway inflammation

1.6.2.1. TH₂ airway inflammation
TH₂ cells are responsible for driving the inflammatory process in allergic eosinophilic asthma (193). Specialized antigen-presenting cells are activated by inhalation of aeroallergens, which then differentiate naïve T-lymphocytes into TH₂ cells. Upstream regulation of TH₂ cell differentiation involves a cascade of events in the airway epithelium regulated by interleukin-33 (IL-33), IL-25 and thymic stromal lymphopoietin (TSLP). Downstream, TH₂ cells release cytokines such as IL-4, IL-5, IL-9 and IL-13. Release of these cytokines leads to activation of airway epithelial cells, chemo-attraction of effector cells (mast cells, eosinophils and basophils), IgE switching in B-cells, airway eosinophilia, mucous hyper-secretion (192) and subsequent airway remodeling.

Non-allergic eosinophilic inflammation can occur and involves activation of TH₂ innate lymphoid cells (ILC2). ILC2s are activated by prostaglandin D2 and epithelium-derived cytokines IL-33, IL-25 and TSLP, which are released when epithelium is damaged by air pollutants, microbes and glycolipids (192, 193, 222). Activated ILC2s also produce IL-5 and IL-13, which results in eosinophilia, mucous hyper-secretion and airway hyper-responsiveness (192, 193) (figure 1.6).

TH₂ and eosinophilic inflammation has long been strongly associated with atopy and allergy, type I hypersensitivity reactions and a good response to steroids (62). This type of inflammation is more common in mild-to-moderate asthma and seen in the majority of people with asthma (10, 223). TH₂
associated asthma can be further classified into phenotypes based on the age of asthma onset (62). Although a specific age-cut off has not been defined, early-onset and later-onset asthma are recognized eosinophilic phenotypes, with the former usually occurring pre-adolescence and the latter after 20 years old. Early-onset TH₂ asthma is characterized by allergic symptoms and atopic disease. Disease severity can range from mild to severe and may be associated, but not always, with TH₂ proposed biomarkers such as IgE, high FeNO, sputum eosinophilia and increased airway periostin (62, 214, 224). It is often responsive to corticosteroid and TH₂-targeted therapy (225-228). Later-onset eosinophilic asthma is characterized by increased eosinophils numbers compared to normal, in either sputum, bronchosscopic or blood samples (>2% often used in sputum samples). In contrast to early-onset allergic asthma, the disease is often severe and associated with sinusitis, nasal polyps or aspirin-exacerbated respiratory disease but not allergic symptoms. Persistent eosinophilia occurs despite corticosteroid therapy (10, 223, 229). The TH₂ process in later-onset asthma probably differs and is more complex than early-onset asthma because of the lack of allergic features (62). Despite the lack of association with allergic features, treatment targeted at a TH₂ related cytokine pathway (IL-5) in late-onset persistent eosinophilic asthma can reduce blood and lung eosinophils, systemic steroid requirements and the number of exacerbations (230).

1.6.2.2. TH₁ and TH₁₇ airway inflammation
Non-eosinophilic inflammation can occur in asthma without the presence of TH₂ cytokines (213, 231). Neutrophilic inflammation may dominate this
process and involves the release of ILC3 and cytokines such as IL-17 by TH\textsubscript{1} and TH\textsubscript{17} cells (218) or type 3 ILCs. This process also activates macrophages with subsequent release of neutrophil chemokines such as CXC motif chemokine ligand (CXCL-8) and further neutrophil recruitment (193) (figure 1.5). These mechanisms are poorly understood however the presence of neutrophils is thought to reflect bacterial colonization (232). Furthermore, treatment with corticosteroids inhibits neutrophil cell apoptosis (233-235) therefore promoting neutrophil survival and may contribute to neutrophil activation. Corticosteroids can also suppress TH\textsubscript{2} immunity, which may result in up-regulation of TH\textsubscript{1} and/or TH\textsubscript{17} immune responses (236).

A neutrophilic asthma phenotype has been associated with adult-onset and severe asthma (10, 206, 237, 238). However there is no clear consensus regarding what level of neutrophilic inflammation defines this phenotype. Neutrophilic inflammation is often seen in asthmatics treated with corticosteroids (239) and as discussed above, could suggest either corticosteroid unresponsiveness or that corticosteroids may play a role in the development of neutrophilia. Increased airway neutrophils are associated with lower lung function, thicker airway walls as measured by computed tomography (CT) scan and increased matrix metalloproteinase (MMP) expression but not with AHR (30, 33, 232, 240, 241). The presence of airway neutrophilia in asthma is controversial, as studies have included smokers or ex-smokers and when smoking status and corticosteroid use are controlled for, this phenotype of neutrophilic asthma does not exist (242, 243).
Additionally, older age may be a confounding factor because of the association with age and airway neutrophilia (28-30, 216).

The role of TH\textsubscript{17} inflammation in asthma is not completely understood however is associated with neutrophilic inflammation (244) and has been implicated in severe asthma. TH\textsubscript{17} cells are a subset of CD4\textsuperscript{+} T cells that release IL-17, which act on epithelial cells to release CXCL-1 and CXCL-8, which are chemokines that attract neutrophils. IL-6 is also released and enhances TH\textsubscript{17} cell activation. IL-17 cytokines are thought to be pro-inflammatory and have been associated with an increase in proteolytic enzymes (neutrophil elastase and MMP-9) in animal studies (245, 246), which could potentially contribute to lung tissue breakdown.

1.6.3. Lung tissue inflammation
Inflammation and changes to the lung tissue is not commonly associated with asthma because it is typically thought of as a disease affecting the airways. Contribution of lung tissue to asthma pathogenesis is not well recognized because evidence is limited and based on a small number of post-mortem and bronchoscopy studies (160, 208, 209, 247, 248). Inflammation and breakdown of lung tissue in asthma may occur as a result of inflammation extending from the outer or adventitial layer of the small airways to the surrounding alveolar walls. This is based on inflammation occurring predominantly in the adventitial layer in the small but not large asthmatic airways (160, 247, 248).

Inflammatory process occurring in the lung or alveolar tissue has also been demonstrated in living stable asthmatics (208). The predominant inflammatory
cells were eosinophils and macrophages and were greater in number in the alveolar tissue compared to airway tissue (208). Inflammation is also accentuated during the night in nocturnal asthmatics, suggesting circadian variation may contribute to lung tissue inflammation (208). Nocturnal decline in lung function is associated with eosinophils in the alveolar but not proximal airway tissue (208). Additionally, in nocturnal asthmatics a greater number of CD4+ lymphocytes were found in alveolar tissue during the night, compared to non-nocturnal asthmatics (209). Alveolar tissue but not airway CD4+ cells, which are cells that can drive eosinophil recruitment, also correlate with nocturnal worsening of lung function (209).

1.6.3.1. Lung tissue breakdown and proteolytic cascade
It has been proposed that bronchiolar inflammation results from recurrent asthma attacks, which in turn activates a pro-inflammatory proteolytic pathway that can cause tissue breakdown (16, 18, 197, 198, 249). This pathway is mediated by an autocrine epidermal growth factor (EGFR) signalling cascade, also initiated by inhalation of aeroallergens or noxious stimuli (249). Protective mucociliary responses occur in the epithelium via IL-17 and IL-18, which activates IL-8 to induce mucin production and promote neutrophil recruitment (198, 249). Mucin production can also be induced by proteases including neutrophil elastase and cathepsin G and by eosinophils, macrophages and mast cells via activation of the EGFR ligand-initiated signal cascade (249). These proteases together with activated MMPs are part of the proteolytic cascade, which could potentially cleave and disrupt terminal bronchiole-lung tissue attachments (249). Consequently lung tissue breakdown may lead to
mild emphysema (18, 198), similar to the terminal bronchiole-lung tissue uncoupling seen in smokers with emphysema (250). A pro-inflammatory proteolytic cascade can explain lung tissue breakdown in both these situations.

Indirect evidence of MMPs contributing to this proposed mechanism of lung tissue breakdown in asthma has been demonstrated in autopsy studies (191). Increased content of MMP-1, MMP-2 and MMP-9 was found in the outer area of the small airways (i.e. areas of the airway wall in close proximity to alveolar tissue) in those who died from asthma compared to non-asthmatics who died from other causes (191). Composition of ECM proteins in the outer airway wall was also altered in the asthmatic patients; fibronectin was increased and collagen III was reduced (191). Additionally, in the peri-bronchiolar lung tissue of the asthmatic patients MMP-9 and collagen I content was increased (191) (figures 1.7 and 1.8). The presence of proteolytic proteases (MMPs) and changes to the outer airway wall in fatal asthmatics could therefore alter the mechanical properties between the airways and lung tissue.
The distal lung is a major site of extracellular matrix (ECM) remodelling in fatal asthma, with an imbalance of collagens I and III and increased fibronectin and matrix metalloproteinase (MMP) content. This diagram summarises the differences in ECM composition in the small airways between healthy controls and patients with fatal asthma. \(\downarrow\): Significantly lower compared with that seen in healthy controls. \(\uparrow\): Significantly higher compared with that seen in healthy controls.
Figure 1.8

From Dolhnikoff et al (191).

Fibronectin and matrix metalloproteinase-9 (MMP-9) content (in square micrometers per micrometer) in the small airways and peri-bronchiolar lung tissue in asthmatic patients and control subjects. IS: inner area of the small airways, OS: outer area of the small airways, PP: peri-bronchiolar parenchyma or lung tissue. The median is represented as horizontal bars.

Fibronectin: *P < 0.02 compared with control subjects. MMP-9: *P < 0.05 compared with control subjects.
There is also contradictory evidence showing MMP-9 is reduced in both airway and lung tissue in asthma compared to healthy controls (188). Although within the asthma group, those with uncontrolled asthma showed increased MMP-9 content compared to the controlled asthmatics (188). Additionally, the amount of tissue-inhibitor of MMP-3 was increased in the asthmatics compared to healthy controls in both airway and lung tissue (188).

Inflammation in asthma is now recognized as being more diverse, with eosinophilic and neutrophilic inflammation often co-existing. The type of inflammation present relates to various clinical features of asthma (62) and importantly, the lack of eosinophilia and response to corticosteroids does not rule out asthma. It is likely that more than one inflammatory cell and pathway are involved in the various asthma phenotypes and contribute to inflammation and alteration to the inflammatory profile over time (222). Inflammation of the lung tissue should not be ignored because it is likely to play a role in airway-lung tissue uncoupling and therefore alter the mechanical function and interdependence (160, 191, 247, 248). The functional consequences of airway and lung tissue changes in asthma will be discussed in the next section.
1.7. Asthma Pathophysiology: Functional changes

Airway remodeling and lung tissue changes in asthma can result in airway narrowing and subsequent variable airflow obstruction (1). Other functional changes that can occur in asthma include AHR, gas trapping and hyperinflation; increased airway resistance and ventilation heterogeneity and altered airway and lung mechanics. These changes can manifest clinically with wheeze, cough, dyspnea and chest tightness.

1.7.1. Airflow obstruction, AHR and hyperinflation

Airflow obstruction is commonly measured and quantified using spirometry (251). Spirometry requires a forced exhalation manoeuvre to derive the indices Forced Expiratory Volume in 1 second (FEV₁) and Forced Vital Capacity (FVC). A reduced spirometric ratio (FEV₁/FVC) and/or bronchodilator reversibility is indicative of airflow obstruction (1, 251). FEV₁ declines with age (119, 120, 145, 252, 253) and is probably attributed to age-related structural airway and tissue changes (105). The degree of airflow obstruction is an important marker of disease severity and useful for prognosis (254-257). Longitudinal studies have demonstrated a more rapid decline in lung function in people with asthma (257), more so if there is a history of smoking (119, 120, 252). Mucous hyper-secretion in asthma is also associated with a faster decline in FEV₁, regardless of the smoking history (252, 258).

Airway hyper-responsiveness (AHR), a typical hallmark of asthma (39), is an exaggerated narrowing of the airway in response to a provoking stimulus (259) and can be measured using a bronchial provocation challenge test (260). AHR is associated with various clinical features of asthma including
disease severity (261, 262), an increased rate of FEV₁ decline (263) and fixed airflow obstruction development (2). AHR may increase the risk of severe asthma exacerbations (264) and may be reduced or even abolished with asthma treatment (265, 266).

Changes in lung volumes such as hyperinflation and gas trapping also occur in asthma (267, 268). Hyperinflation can be measured using body plethysmography and demonstrated by an increase in functional residual capacity (FRC). Increased FRC may occur during an exacerbation and be transient or can persist despite treatment improving airflow obstruction (20, 268-275). The degree of hyperinflation during acute asthma exacerbations may correlate with the severity of airflow obstruction (267, 268, 276) and FRC may increase to volumes as high as the baseline total lung capacity (TLC) (267, 268). Gas trapping can be determined by an increase in residual volume (RV) or RV/TLC (277, 278). Airway closure and prolonged expiratory time constants are thought to contribute to hyperinflation and gas trapping (276). Persistent inspiratory muscle contraction during expiration in the presence of bronchospasm (276, 279) and loss of lung elastic recoil are also thought to play a role (269, 274, 280, 281).

1.7.2. Small airways
The respiratory or traceho-bronchial tree is made up of dichotomously branching airway structures divided into approximately 23 airway generations (282, 283) (figure 1.9). The proximal airways, made up of the first 15 generations, constitute the anatomical dead space and are referred to as the conducting zone or airways (282). From generation 16 onwards are the
respiratory bronchioles, alveolar ducts and sacs. These are referred to as the transitional and respiratory zones and make up the acinar airways where gas exchange occurs (282). As the airway generation advances, the number of airways increases exponentially, however both the airway length and diameter get smaller (282). The velocity of air flow and gas transit within the lung also changes and transitions from bulk convective flow in the conducting airways to diffusion down a concentration gradient in the acinar airways (282). With successive airway generations the total airway cross-sectional surface area increases dramatically and as a result resistance to airflow decreases (282).
Figure 1.9

From Weibel et al (283).

Tracheo-bronchial tree made up of 23 airway generations.
Also known as the distal or peripheral airways, the small airways are the main site of airflow obstruction in asthma (14, 15, 284, 285). The small airways correspond to airway generations 8-23, are less than 2 mm in diameter and do not contain cartilage in their walls (283). In healthy lungs, resistance to airflow in the small airways is low because they make up a large cross-sectional surface area however only contribute to 10-20% of total airway resistance (285). Therefore the small airways have been coined with the term ‘silent zone’ (284) because airflow obstruction or small airway dysfunction is not easily identified until disease has affected a significant portion of the small airways.

Conventional lung function tests such as spirometry may miss early or mild disease in the small airways, thus more sensitive methods to detect abnormalities early are required. Currently available tests that may be more sensitive than spirometry (286-291) include the multiple breath nitrogen washout test (292) and forced oscillation technique (293). Both tests are performed at tidal breathing and do not require a forced expiratory manoeuvre. Therefore these tests may be more attractive than spirometry, as they are unlikely to alter smooth muscle tone because a forced expiratory manoeuvre is not used (294). These tests will be discussed in more detail later in this section.

1.7.3. Small airway dysfunction
Small airway dysfunction has been measured using indices derived from the FVC manoeuvre such as the forced expiratory flow at 25-75% of FVC (FEF25-75%) (295). However, the FEF25-75% may not be clinically useful (296) and its
reproducibility and sensitivity has been challenged (297, 298). A reduced FEF_{25-75%} measurement, in the presence of normal spirometry, is thought to reflect ‘noise’ or be due to reduced lung volume rather than airways disease (296). There is also a poor correlation between FEF_{25-75%} and other markers of small airway dysfunction such as gas trapping (7).

Small airway dysfunction can also be characterized by the amount of ventilation heterogeneity (VH) in the lungs (292), in other words assessing the unevenness or efficiency of gas mixing within the lungs (299, 300). A degree of uneven gas mixing exists in healthy lungs because of airway structure asymmetry, anatomical inter-lobar differences and gravitational effects between the lung apex and base (282, 283). Differences in the lungs resistive and elastic properties also contribute to sequential filling and emptying of the lung (301, 302). This results in variation in time constants for gas emptying, where better-ventilated lung units have a preference to empty faster than less well-ventilated lung units (302). Hence alterations to the lungs mechanical properties (i.e. the resistive and elastic properties) that occur in disease such as asthma can also increase VH because small airway obstruction can increase airflow resistance.

1.7.4. Ventilation Heterogeneity

1.7.4.1. Multiple Breath Nitrogen Washout

VH can be measured using the multiple breath nitrogen washout (MBNW) (292) test, a non-invasive lung function technique that was first described in 1950 (299). The MBNW test provides information about the degree of VH or efficiency of gas mixing within the lungs, possible mechanisms behind
abnormal ventilation distribution and the location of underlying pathological processes (292). The test involves measurement of the expired concentration of an inert tracer gas (i.e. nitrogen, N₂) at the mouth, which is washed out by inhalation of 100% oxygen (O₂) over a series of tidal breaths. If VH is increased (i.e. abnormal) there is a delay in clearance of the tracer gas therefore more breaths will be required to ‘washout’ the N₂. The MBNW test can also assess lung volume (FRC), gas trapping and measures of anatomical and respiratory dead space (303).

1.7.4.1.1. MBNW Indices
The anatomical and physiological basis of the MBNW test is centered on the principles of gas transport and mixing (304). Compartimentalization of VH distribution (292, 305-307) allows indices to be derived, which reflect VH or specific ventilation (ventilation per unit lung volume) within either the conducting or acinar airways (308). Uneven ventilation or differences in specific ventilation in lung units larger than the acini (i.e. the more proximal airways), where gas transport occurs by convection, is termed convection-dependent inhomogeneity (CDI) (308). In the more distal airways where gas transport occurs by diffusion, uneven ventilation in this lung region is termed diffusion-convection-dependent inhomogeneity (DCDI) (308). Relative contributions to gas mixing from interactions between the convection- and diffusion-dependent airways occurs in an intermediate zone and form what is called the ‘diffusion-convection front’ (304). This quasi-stationary diffusion-convection front determines where CDI and DCDI mechanisms operate and is thought to arise around the acinar entrance in healthy adult lungs (309).
VH arising from CDI and DCDI mechanisms are expressed as the clinical indices Scond and Sacin (306). These indices have been derived from theoretical (310), experimental (311) and lung modeling (305, 312, 313) analyses of MBNW normalized phase III slopes, which are described in more detail below and in chapter 2. Although the exact anatomical location of these indices are not known, Scond is thought to be affected by the mechanical properties of the lung units that are larger than the acini (i.e. in the conducting airways). Sacin on the other hand is thought to arise due to heterogeneity of the cross-sectional areas of airway openings in the terminal airways and the acini (313) (figure 1.10). A global measure of VH can also be derived from the MBNW test, lung clearance index (LCI), which describes the lungs overall gas mixing efficiency. An increase in Scond, Sacin or LCI indicates increased VH in the conductive-dependent airways, diffusion-dependent airways or poor overall gas mixing efficiency, respectively.
Figure 1.10

From Verbanck et al (313).

Schematic representation of predicted changes of normalized phase III slopes versus lung turnover or breath number and corresponding changes in MBNW indices Scond and Sacin, following structural alterations in the proximal or the lung periphery.
1.7.4.1.2. Expirogram and phase III slopes
The MBNW expirogram or washout curve is obtained by measuring N₂ concentration at the mouth over consecutive breaths after 100% O₂ is inhaled. The washout curve exhibits 4 distinct phases with the shape of the curve reflecting both anatomical (i.e. dead space) and physiological properties associated with gas mixing in the lungs. The N₂ washout curve phases can be described using the single breath N₂ washout test (figure 1.11) (292). Phase I reflects the dead space that contains pure O₂; phase II is the ‘bronchial phase’ where N₂ concentration increases rapidly as the gas exchanging units start to empty sequentially; phase III is the ‘alveolar phase’ and is the key MBNW parameter, as the slope represents differences in N₂ concentration between gas exchanging units as they continue to empty. The phase III slope is usually calculated between 25% and 75% of the expired volume to avoid contribution from phase IV. Phase IV shows a rapid increase in the expired N₂ concentration and reflects the onset of airway closure in the dependent portions of lung at lower lung volumes.
Figure 1.11

From Robinson et al (292).

Example of a typical single-breath washout (SBW) trace. Nitrogen gas (N$_2$) expirogram showing calculation of phase III slope (SIII) between 25% and 75% of the expired volume, to avoid the contribution of phase IV. The four phases of the expirogram are also demonstrated: phase I (absolute dead space), phase II (bronchial phase), phase III (alveolar phase) and phase IV (fast rising phase at end of expiration). $V_{T,exp}$: expired tidal volume.
If ventilation were even between all gas-exchanging units, the phase III slope would be flat, as N₂ would be washed out evenly or homogeneously. However, the normal lung has different compartments that fill and empty at different rates, therefore there is always some unevenness in ventilation and hence the phase III slope cannot be flat. A change in the phase III slope indicates a change in the distribution of ventilation (i.e. N₂ washes out from different lung units at slower rates) and becomes steeper with disease indicating more uneven or heterogeneous ventilation.

1.7.4.1.3. MBNW equipment
The MBNW set up requires equipment to measure flow, the ability to deliver and allow inhalation of 100% O₂, a gas analyser and a computer and software to record and process data (292). Subjects inhale 100% O₂ which ‘washes out’ N₂. N₂ concentration is measured at the mouth and subjects continue to inhale 100% O₂ until alveolar N₂ is reduced to 1/40th of the initial concentration (figure 1.12). Measurement of both flow and inert tracer gas (N₂) concentration should be synchronized accurately and precisely to minimize measurement error (292).
Figure 1.12

From Robinson et al (292).

Example of a typical multiple-breath washout (MBW) trace. Time series display of (a) volume and (b) nitrogen gas (N₂) from a N₂ MBW test. The volume trace shows stable 1 litre tidal breathing.
The MBNW technique has evolved over the years and equipment has become more sophisticated with advances in technology (292). Reference equations for predicted values exist (287, 314-316), although a standardized reference equation is lacking, in part because of variations in equipment (317), technique, analysis and upper age limit in the populations studied. However, consensus guidelines have been established with recommendations for equipment specification, validation and measurement techniques (292). Differences between currently available MBNW equipment (hardware and software) will be discussed in chapter 2 (317).

1.7.5. Ventilation heterogeneity in asthma
VH contributes to the clinical manifestations and underlying pathophysiological mechanisms in asthma. VH is increased in asthma (315, 318, 319) and more so during exacerbations (318). VH indices are associated with different clinical features of asthma, such as AHR (113), response to bronchodilator (319) and inhaled corticosteroid therapy (320, 321) and asthma control (321, 322). In both stable and unstable asthma (i.e. during asthma exacerbations), VH in the diffusion-dependent airways (Sacin), yet not Scond, is correlated with FEV$_1$ (318), suggesting the acinar airways play a significant role in airflow obstruction in asthma (318).

VH also increases with age (314, 316), with an accelerated increase seen in Sacin and LCI after the age of 60 years, as opposed to a steady linear increase in Scond (314). This may explain some of the differences seen in VH between young and older people with asthma (113). Sacin is higher in older compared to younger asthmatics, despite a similar FEV$_1$ (113), which
suggests the underlying mechanisms in asthma may vary with age. In young adults with asthma, VH in the conductive-dependent airways (Scond) is a major determinant of AHR. Conversely, in older people AHR is predicted by VH in the diffusion-dependent airways (Sacin) (113), suggesting the acinar rather than conductive airways contribute to VH in older people with asthma.

1.7.6. Respiratory Impedance

1.7.6.1. Forced Oscillation Technique
The forced oscillation technique (FOT) is another non-invasive lung function tool that can measure small airway function by assessing the mechanical properties of the respiratory system (294). FOT was first described by DuBois in 1955 (323) and involves external sinusoidal oscillations being applied to the lungs during tidal breathing (294). The external oscillations are small pressure signals generated by a loud speaker and are of varying frequencies that are higher than the tidal breathing rate, upon which the oscillations are superimposed. This allows respiratory impedance (Zrs) (323), the resistive and elastic properties, to be measured and therefore provides information about the lungs’ mechanical properties.

1.7.6.1.1. FOT Indices
Respiratory impedance encompasses the properties of flow and pressure and describes their relationship with respect to a frequency domain (324). Mathematical modelling has allowed partitioning of the contributions from the airways and lung tissue to respiratory impedance. The different properties of the airways and lung tissue allow them to exhibit distinct relationships with different oscillation frequencies (324, 325).
Respiratory impedance is comprised of two components – resistance ($R_{rs}$) and reactance ($X_{rs}$) (294). $R_{rs}$, the in-phase or ‘real part’, describes the resistance of airflow through the respiratory system and reflects airway caliber (294). $X_{rs}$, the out-of-phase or ‘imaginary part’, describes the elastic properties or oscillatory stiffness of the respiratory system and incorporates the concepts of inertance and capacitance. Inertance refers to the mass-inertive forces of air moving in the conductive airways and dominates at higher oscillation frequencies. Whereas capacitance refers to the elastic properties of the lung periphery and dominates at lower oscillation frequencies (294). $X_{rs}$ can be thought of as reflecting airway closure and subsequent unevenness of VH; if more airways are closed the lungs will appear more ‘stiff’ because the applied oscillation signals cannot reach the peripheral lung (294). The concept of $X_{rs}$ is not widely understood because of the complexity of the parameters however it is important to consider both $R_{rs}$ and $X_{rs}$ in interpreting respiratory mechanical properties (294).

FOT allows simultaneous measurement of $R_{rs}$ and $X_{rs}$ over a range of frequencies (usually from 3-35 Hz) (294). It is thought that measurements over higher frequency signals (>20 Hz) do not reach the small airways therefore reflect contribution from the larger airways (326). This is because signals at higher frequencies are absorbed by the respiratory system before reaching the small airways (294, 326, 327). Measurements over lower frequencies (<15 Hz) reflect contribution from the small airways because these signals penetrate deeper into the lungs (294, 326). However, there is no clear consensus on which frequency range best reflects small airway function.
and the anatomical location of the transition between the small and large airways is yet to be determined (326).

At frequency ranges between 4-10 Hz, which reflects mainly resistance of the airways, the healthy respiratory system demonstrates frequency independent $R_{rs}$. In other words, resistance changes very little with increasing or varying frequency (328, 329). In contrast to $R_{rs}$, $X_{rs}$ is frequency dependent. That is, $X_{rs}$ is negative at low frequencies, where lung and chest wall elastance dominate and becomes more positive at higher frequencies, where airway inertia begins to dominate (329). The frequency at which $X_{rs}$ crosses zero (i.e. when $X_{rs}$ transitions from negative to positive) is called the resonant frequency and occurs when the elastic and inertial forces are equal and opposite in magnitude (figure 1.13). In healthy lungs resonant frequency is thought to occur between 8-10 Hz (293).
Figure 1.13
From Oosteveen et al (293).

Frequency dependence of (a) resistance and (b) reactance in health (solid lines) and obstructive airway disease (interrupted lines) in adults. Arrow indicates resonant frequency.
1.7.6.1.2. FOT Equipment
The FOT set up requires equipment that enables a route to measure flow and pressure and another route where external pressure signals or oscillations can be applied to the lungs. A computer and software are connected to these two routes and used to generate signals, record and process data (figure 1.14). The external oscillations can be mono- or multi-frequency and are delivered as a continuous sinusoidal signal (pseudo-random noise) (293, 294). Both in-house and commercial FOT devices are in use and utilize different hardware and software, however the general principle of the technique remains the same. Attempts to standardize the technique have been made with recommendations in the consensus guidelines (293). Predictive values for the indices measured by FOT are also available, however these values are limited to the Caucasian population (293, 329).
Figure 1.14

From Navajas et al (330).

Schematic diagram showing the setup for measuring respiratory impedance.
1.7.7. Resistance and reactance in asthma

In both adult and paediatric asthma, including during stable states (331), $R_{rs}$ is increased or higher than normal and exhibits frequency dependence (i.e. increased $R_{rs}$ occurs predominantly at low frequencies and subsequently reduces with increasing frequency) (289, 331-333). In patients with respiratory symptoms and a normal FEV$_1$, $R_{rs}$ is increased (289) suggesting FOT may be a sensitive tool for discriminating between disease and health. $R_{rs}$ is also comparable to plethysmographic or oesophageal pressure techniques used for estimating resistance to breathing (333), indicating $R_{rs}$ as measured by FOT could be used as an alternate technique to determine airway resistance.

$R_{rs}$ may be normal in asthma and can increase following a histamine challenge test (328). Frequency dependence of $R_{rs}$ is demonstrated post histamine challenge only in asthmatics and not healthy controls, with the increase in resistance being most marked at lower frequencies (328) (figure 1.15). The addition of FOT to assessment of AHR can therefore provide information about localization of the response in the respiratory system (328).
Figure 1.15
From Wouters et al (328).

Top panel: Mean respiratory resistance \((R_{rs}, \text{kPa(L/s)})\) before (closed circles) and after (open circles) histamine challenge in healthy controls and asthmatics.

Bottom panel: Mean respiratory reactance \((X_{rs}, \text{kPa(L/s)})\) before (closed squares) and after (open squares) histamine challenge in healthy controls and asthmatics.
\( X_{rs} \) may be abnormal in asthma, with values being more negative compared to normal (289, 328, 334). Similarly to \( R_{rs} \), following a histamine challenge test \( X_{rs} \) becomes more abnormal – it decreases or becomes more negative at all frequencies (328) (figure 1.15). This is probably due to the presence of more peripheral airflow obstruction and oscillation signals subsequently not reaching the lung periphery. \( X_{rs} \) can also be partitioned into inspiratory and expiratory \( X_{rs} \) values at 5Hz. This may allow differentiation between COPD and asthma, which can be a challenge when \( \text{FEV}_1 \) is similar between the two obstructive airway diseases (335). The difference between inspiratory and expiratory \( X_{rs} \) is greater in COPD compared to asthma and thought to be due to enhanced dynamic airway narrowing in COPD (335). This probably reflects the contribution of lung tissue (i.e. emphysema) and loss of elastic recoil that occurs in COPD.

1.7.8. Lung tissue mechanics – elastic recoil and compliance
Alteration to the lung tissue can occur in asthma (16-18) and may contribute to the functional changes described above because of changes in the lungs elastic recoil properties. Lung elastic recoil can be described as lung compliance or the ease with which the lungs can expand or stretch.

The lung is a complex 3-dimensional structure enclosed within the thoracic cage, which encompasses the respiratory muscles and chest wall. The lung is separated from the chest wall by the pleural space and has a tendency to recoil inwards. The chest wall on the other hand has a tendency to recoil outwards. Negative pressure within the pleural space prevents the lungs from
collapsing. The lungs (alveoli) are also held open by a balance between tissue forces and alveolar surface tension (186).

1.7.8.1. Elastic recoil measurement
Lung compliance is defined as the change in lung volume for any given applied pressure. Static compliance represents lung compliance during periods of no airflow, such as during an inspiratory pause. Dynamic compliance refers to lung compliance during periods of airflow (i.e. during inspiration).

To measure compliance and the lungs elastic recoil properties, the pressure or force required to drive air in and out of the lungs and the volume or amount of air being driven needs to be measured. The distending pressure across the lung (trans-pulmonary pressure, $P_L$) determines the volume of the lung. Therefore changes in the distending pressure determine changes in lung volume. The distending pressure of the lung itself ($P_L$) is equal to the difference between the pressure inside the lung or alveolar pressure ($P_{ALV}$) and pressure in the pleural space ($P_{PL}$). At the end of a normal breath (i.e. at FRC) when there is no airflow, $P_{ALV}$, pressure at the mouth ($P_{AO}$) and atmospheric pressure ($P_{ATM}$) are equal. Therefore $P_L$ can be derived easily when there is no airflow because $P_{ALV}$ is zero (figure 1.16).
Figure 1.16
From Benditt et al (336).

Schematic showing equations for trans-pulmonary ($P_L$), trans-chest wall ($P_{CW}$) and trans-respiratory system ($P_{RS}$) pressures. $P_{PL}$: pleural pressure, $P_{ATM}$: atmospheric pressure, $P_{ALV}$: alveolar pressure, $P_{AO}$: pressure at the airway opening.
The trans-pulmonary pressure or pressure across the respiratory system can be derived by measuring the pleural pressure. A surrogate measure of the pleural pressure can be obtained by measuring the oesophageal pressure, using an oesophageal balloon placed in the lower third of the oesophagus (337, 338). In this position the oesophagus is adjacent to the pleural space. Volume is derived by simultaneous measurement of airflow at the mouth. A static (or quasi-static) pressure-volume (P-V) curve can be constructed using these pressure and volume measurements. Static compliance can be derived from the P-V curve by measuring the chord shape of the linear portion of the curve from FRC plus 0.5-1 L (19, 339) (figure 1.17). An exponential function (340) can also be fitted to the P-V curve to derive indices, which describe and provide objective measures of the lungs elastic properties. The exponential function is thought to provide a better measurement of the lungs elastic recoil properties than the static compliance measurement because it incorporates the volume over a meaningful range and does not linearize a curvilinear relationship (340) (figure 1.18). Details of this measurement will be discussed in chapters 3 and 4.
Figure 1.17

Courtesy of Norbert Berend presentation.

Schematic showing calculation of static compliance ($C_L$) from the pressure-volume curve. Vol: volume, $P_L$: trans-pulmonary pressure.
Figure 1.18

Schematic showing the pressure-volume (P-V) curve fitted with the Colebatch equation \( V = A - B \exp(-KP) \) to derive indices reflecting the lungs elastic properties. \( V \): volume as % of total lung capacity (TLC); \( P \): trans-pulmonary pressure; and \( A, B \) and \( K \) are constants. \( A \): is the horizontal asymptote of the Y-axis, \( B \): is the distance on the Y-axis between \( A \) and the extrapolated Y-axis intercept of the curve.
Within the pleural space and lungs there is also a pressure gradient, whereby the pleural ($P_{PL}$) and alveolar pressures ($P_{ALV}$) change between the apex and base of the lung. The pressure at the apex is more negative than that at the base because of gravity. $P_{PL}$ increases by about 0.25 cmH$_2$O per centimetre of vertical distance from the apex to the lung base (1.19). Therefore $P_{ALV}$ and $P_{RS}$ also changes from apex to base and at rest, alveoli at the top of the lung are more fully expanded than those at the bottom at FRC. Thus different alveoli operate on different portions of the P-V curve, with alveoli at the bottom expanding more than those at the apex, as they are better ventilated. At TLC, although the pressure gradient still exists, theoretically all alveoli are the same size but are operating on the flat portion of the P-V curve.

![Diagram showing pleural pressure gradient](image)

**Figure 1.19**

Courtesy of Norbert Berend presentation.

Schematic demonstrating the pleural pressure gradient, where pleural pressure is more negative at the apex compared to the base.
1.7.9. Loss of lung elastic recoil
Changes to the lung tissue may result in a loss of lung elastic recoil and/or increased lung compliance (18, 341). Loss of elastic recoil can occur in asthma and has been demonstrated objectively using the P-V curve (19-21, 117, 342) (1.20). A leftward shift in the P-V curve and/or a steeper curve indicates a loss of elastic recoil and/or increased compliance (16, 18-21, 117, 343). Loss of elastic recoil leads to a reduction in the driving pressure during maximal expiratory flow ($V_{\text{max}}$) (117) therefore reduced expiratory airflow. The tethering force of the lungs, which holds the airways open, is also reduced therefore leads to airway instability and airways that are prone to premature closure and collapse (274, 344). Premature airway collapse leads to a reduction in $V_{\text{max}}$ at a given lung volume (345, 346). It has also been speculated that a chronic reduction in expiratory $V_{\text{max}}$ may be due to early loss of lung elastic recoil (117). The underlying mechanisms are not clear however micro-emphysema has been suggested as a cause of loss of elastic recoil in non-smokers with asthma (16, 18). In the absence of emphysema, another simple explanation is difficult to determine, however stress relaxation has also been implicated (347-349).
Figure 1.20

From Finucane et al (19).

Mean pressure-volume curves for emphysema, asthma and healthy controls corrected for variation in lung size by expressing lung volume as a percentage of the predicted total lung capacity (TLC). The emphysema curve is steeper and leftward shifted indicating increased compliance and loss of elastic recoil. The asthma curve is leftward shifted indicating loss of elastic recoil.
Surrogate measures of lung elastic recoil may include measures of lung volume (hyperinflation or an increase in FRC or TLC) and gas trapping (increase in residual volume) (269, 350). An increased TLC can occur during severe asthma attacks and is thought to be a physiologic marker of loss of lung elastic recoil (350, 351). Treatment of acute asthma attacks can improve both an increased TLC (267, 268, 273, 284, 351) and loss of lung elastic recoil, implying loss of lung elastic recoil may be reversible (350, 352). Optimization of asthma control in long-standing asthma may also improve the loss of lung elastic recoil (18). On the other hand, loss of lung elastic recoil may be irreversible and is thought to reflect the growth response to hyperinflation during growing years (342) or reflects the response to sustained inspiratory efforts (353). Irreversibility may not be the correct term to describe the lack of change in lung elastic recoil, however in stable asthma lung elastic recoil does not change or improve post bronchodilator therapy (21).

Elastic load provided by the lung tissue is transmitted to the airways through alveolar attachments (354), which are attached to the adventitia or outer airway wall (355). This creates mechanical airway-lung inter-dependence, which is important for maintaining a patent airway lumen. Airway-lung tissue interdependence can be lost due to uncoupling of airway-lung attachments and the consequences are similar to what is described above with the loss of lung elastic recoil. Airways become more unstable and prone to closure (356) and collapse (274, 344). This mechanism may also explain the loss of deep inspiration bronchodilation in spontaneous bronchoconstrictive episodes (357).
The mechanisms causing the functional changes described above may be similar to what is seen in smokers without emphysema (250). Compared to non-smokers, smokers without emphysema have abnormal and a reduced number of alveolar attachments (250). These structural changes were associated with inflammation in the small airways and loss of elastic recoil. Loss of alveolar attachments could therefore represent early stages of lung tissue destruction and could explain the loss of elastic recoil. As discussed in the previous section, lung tissue and the outer airway wall are altered in asthma. Hence changes such as alveolar detachment (17), micro-emphysema (18), loss of elastic fibre integrity (17, 176) in the airway wall and inflammation and/or airway remodeling in the outer airway wall appear to contribute to asthma pathogenesis. Therefore it is not unreasonable to suggest asthma is a disease of both the airways and the lung tissue. Involvement of lung tissue may underpin the mechanisms of FAO in asthma, which will be discussed in the next section.
1.8. Fixed Airflow Obstruction in Asthma

Asthma with irreversible or fixed airflow obstruction (FAO) (3, 5, 8, 358-362) is a recognized asthma phenotype that can develop despite appropriate treatment (252, 253). Consistent findings show that inhaled corticosteroids are beneficial for asthma control, reducing exacerbations and airway inflammation and improving lung function (228, 363). However corticosteroids are not effective at preventing FAO in some patients. FAO is typically associated with COPD, which is usually due to cigarette smoke or exposure to other noxious particles such as occupational dust, chemicals, biomass and coal cooking (364). However unlike COPD, FAO in asthma can occur despite no or minimal smoking history (5, 85).

FAO in asthma is associated with poor outcomes and has been classed as a sub-type of severe or refractory asthma (365, 366). In addition to being associated with moderate to severe disease (2, 5, 367, 368), individuals with asthma and FAO have worse lung function, a faster decline in lung function, more frequent exacerbations and poor quality of life (4, 5, 7-10). The presence of FAO also has implications on mortality, as post-bronchodilator FEV₁ has been shown to be a predictor of asthma-related (369) and overall mortality (370).

Current evidence on FAO in asthma is largely based on a small number of longitudinal studies and cross-sectional studies (2, 5, 18, 33, 358, 362, 367, 371, 372). However the disease is highly variable and heterogeneous, therefore only limited insight can be gained from cross-sectional data.
Identifying those at risk of developing FAO is challenging due to many unknowns about the underlying pathophysiology.

1.8.1. Definition and Prevalence of FAO
A standardized definition for FAO in asthma is lacking despite irreversibility in asthma being recognized for decades (3, 359, 360, 362, 373). The absence of a standardized definition may be due the lack of a clear consensus on what constitutes reversibility in airflow obstruction (251, 374, 375). This may partly be due to there being no consensus on how to express a bronchodilator response, the variables that should be used and the type, dose and inhalation mode of the bronchodilator agent (251). Nevertheless, a bronchodilator response is usually expressed as either the percent of the initial spirometric value, percent of the predicted value and/or the absolute change (251). Published guidelines recommend using the percent change from baseline and absolute changes in FEV\textsubscript{1} and/or FVC when assessing for a positive bronchodilator response. An improvement in FEV\textsubscript{1} and/or FVC by >12\% and >200ml compared to baseline suggests a ‘significant’ bronchodilator response (251).

Various descriptions and lung function parameters have been used to define FAO. The definition can be tainted by the inclusion of current smokers (368) and/or ex-smokers (376, 377). With respect to the defining parameters, studies have used percent predicted post-bronchodilator spirometric ratio (378) with FEV\textsubscript{1} (2, 117, 358, 379) or FEV\textsubscript{1} alone (3-5, 368, 372). Lung volume parameters such as TLC (2) have also been incorporated into the FAO definition, as a decrease in hyperinflation post-bronchodilator may also
indicate a significant bronchodilator response (380). The cut off values for spirometric ratio and/or FEV\(_1\) also varies between studies. A post-bronchodilator spirometric ratio of <75% (379) is commonly used (2, 117, 379), however some studies have used the lower limit of normal (365, 378) as the cut off. In addition, the percent predicted pre or post bronchodilator FEV\(_1\) cut off varies from as low as <50% up to <70 or <85% (2, 4, 5, 117, 358, 372, 381) and the percent change in FEV\(_1\) cut off from <9% to <15% (5, 358, 381). The type of bronchodilator medication used, dose and route of administration is also not consistent (382-386). Salbutamol is frequently used as the bronchodilator agent, however other agents such as albuterol and ipratropium bromide have also been used (382, 386). Theophylline and oral corticosteroids have also been used in attempt to achieve maximal lung function (3).

Discrepancies in the definition of FAO in asthma make it difficult to estimate the true prevalence. Therefore the reported prevalence of FAO in adults is highly variable. It has been reported to be 23% in a non-smoking asthmatic Danish population (5) followed over a period of 10 years. Other studies have reported a prevalence of 16% in mild asthma (358), up to 49% in severe asthma (2) and in the range of 35-50% in the adult asthmatic patients (63). A recent meta-analysis estimated the incidence of FAO in non-severe asthma patients to be between 8-16% (381).
1.8.2. Risk factors
A number of risk factors have been linked to the decline in lung function and
development of FAO in asthma including a lack of ICS treatment (387) and
chronic mucus hyper-secretion (252, 258).

An irreversible component to asthma may occur early in life, as loss of lung
function can occur at a young age (388, 389). This may be because early lung
insult while lungs are still maturing and growing may predispose the airways
to remodelling and irreversible damage. If the initial loss of function occurs at
an early stage, further decline over the years may then be minimal (390). Low
FEV₁% predicted (391), AHR (391), wheezy bronchitis and asthma in
childhood (392) are associated with an increased risk of reduced lung function
in adulthood.

A limited number of prospective longitudinal studies have demonstrated an
association between FAO and lung function at enrolment (5, 258, 358). FAO
was associated with lower FEV₁, less bronchodilator reversibility and less
severe AHR in adult asthmatics after 26 years of follow up (358). In these
adults with FAO, they were also found to be using less corticosteroids at
follow up (358). In contrast, a greater degree of AHR in childhood was
associated with an increased risk of FAO in adulthood (371, 388, 393).
Furthermore, another study in non-smoking adults with asthma showed a
higher degree of bronchodilator reversibility and long term oral corticosteroid
treatment was associated with FAO after 10 years of follow up (5).

Older age (367, 394) has been associated with FAO development, as has the
age of onset, duration and severity of asthma (3, 4, 359, 372), although the
evidence is also conflicting. Adult-onset asthma is associated with more rapid decline in lung function (2, 229, 395), increased AHR (2) and increased risk of FAO. Yet in another study, adult-onset disease was not related to disease severity, disease duration or lung function impairment (396).

Male sex has been associated with the development of FAO and thought to reflect different gender-specific driving factors (367, 372, 394, 397, 398). On the other hand severe asthma is more commonly seen in females (399). This may be related to ICS having less of an effect on lung function decline in females, both in the short-term and long-term (400, 401). Female sex hormones, differences in airway geometry and deposition of ICS may explain the gender differences in ICS response (366, 402), therefore possibly contributing to FAO development.

Atopy and allergic symptoms are not diagnostic of asthma however they often co-exist and may have a protective effect against FAO (367, 381). Atopic dermatitis, rhino-sinusitis (368, 388) and atopy have been shown to have a reduced risk of FAO in asthma (381). A family history of atopic dermatitis, pets in the home and dust sensitivity also have a protective effect against FAO (367). The mechanism leading to a protective effect is unknown however suggested to be due to influences of characteristic ‘twitchy’ airways on airway structure, mechanical force, inflammation and related repair (367). Aspirin sensitivity on the other hand is associated with an increased risk of FAO (367).

Ethnicity and genetics are thought to play a role FAO developing in asthma. Black ethnicity (367, 394) is associated with an increased risk of FAO, yet
Hispanic ethnicity appears to be protective (367). These ethnic differences suggest genes contributing to asthma susceptibility may vary between races. Specific genes such as ADAM33 have been implicated in asthma development and progression and thought to play a significant role in airway remodelling (55). However the role of the ADAM33 gene and other genes in FAO pathogenesis is unknown.

Lung function declines at an accelerated rate in asthma (5, 256, 257), even more so in smokers (119, 252) and in those with FAO (8) (figures 1.21 and 1.22). Unsurprisingly a history of smoking is associated with an increased risk of FAO (367, 377, 381, 403) and may be related to smoke-induced inflammatory changes (404). It could be argued that in studies that include smokers, some subjects have predominantly COPD rather than asthma. It is likely that the synergistic effect of cigarette smoke combined with asthma contribute to an increased risk of FAO. A recently published study has demonstrated two distinct asthma phenotypes in smokers with low FEV$_1$/FVC (88). One group had features characteristic of TH$_2$ inflammation, what is traditionally associated with asthma. The other group of smokers had low levels of circulating eosinophils (88). The authors suggest different biological pathways contribute to FAO in smokers with asthma.

Chronic or recurrent respiratory infection with specific pathogens may play a role in FAO development in asthma (405, 406). Seropositivity to *Chlamydia pneumoniae* infection has been associated with asthma and may promote FAO development in non-atopic people with adult-onset asthma (405). *C. pneumoniae* is also associated with lower FEV$_1$ (405) and can accelerate the
loss of lung function in non-atopic people with a new diagnosis of asthma (406).

Occupational exposures may be linked with an increased risk of FAO in asthma. Ongoing exposure to agents causing asthma is associated with poor prognosis and faster decline in lung function (407). Removal from the occupational exposure can result in either no recovery or complete recovery of impaired lung function (407, 408).
Figure 1.21

From Lange et al (252).

Changes with age in height-adjusted forced expiratory volume in one second (FEV₁) according to sex, smoking status and the presence or absence of asthma. Lung function declines with age, more so with the presence of smoking.
Lung function measured at two time intervals, 10 years apart, in non-smoking adults with asthma. Lung function declines more rapidly in those with fixed or non-reversible airflow obstruction. FEV\textsubscript{1} % pred: percent predicted forced expiratory volume in 1 second, NRAO: non-reversible airflow obstruction, RAO: reversible airflow obstruction.

Figure 1.22
From Ulrik et al (5).
1.8.3. Mechanisms
FAO in asthma adds an additional complexity to this already heterogeneous disease. The mechanisms leading to FAO are largely unknown and there is limited data assessing both structural and functional changes together in this phenotype. The processes that may contribute to FAO include airway remodelling and/or lung tissue changes (18, 163), which have both been discussed earlier in this chapter.

1.8.3.1. Response to glucocorticosteroids
ICS has demonstrated a beneficial effect on lung function decline (401, 409-411). However this is not a consistent finding as the response to ICS in asthma can be variable or non-existent and may not necessarily prevent FAO from occurring (2, 3, 362). Glucocorticosteroid receptor abnormalities (412) may contribute to the lack of response to ICS.

FAO could potentially be due to airway remodelling being an irreversible process, which may be related to therapy, specifically glucocorticosteroid duration and dose. How glucocorticosteroids affect the different components of the airway wall is largely unknown and therefore the effect on airway remodelling is also unknown. Nevertheless, prolonged ICS treatment can reduce reticular basement membrane (RBM) thickening (413, 414), in conjunction with lung function improving (413, 414). Whereas lower dose ICS and/or shorter treatment periods may have no effect on RBM thickening (413, 415). A study using computer tomography (CT) imaging showed partial reversibility of airway wall thickness with ICS treatment (416). This was thought to reflect either a steroid-responsive component contributing to
inflammatory processes and a steroid-unresponsive component contributing to remodelling processes, both leading to airway wall thickening (416). The fact that FAO can develop despite steroid therapy suggests steroids may have a minimal effect on airway remodelling.

1.8.3.2. Inflammation
The role of inflammation in FAO development in asthma is unclear, as both eosinophilic (2, 372, 417) and neutrophilic (33) airway inflammation are associated with an increased risk of FAO. Exhaled nitric oxide, a surrogate marker of eosinophilic inflammation (381), has been shown to be associated with FAO. Serum periostin, also thought to be related to eosinophilic inflammation, was strongly associated with FAO in well-controlled asthmatics on ICS treatment a recent study (418). In contrast, blood eosinophils and FeN0 levels were not (418). Sputum but not peripheral blood eosinophilia in non-smoking adults with asthma was also associated with FAO, despite treatment with ICS/LABA for more than one year (2). This suggests refractory airway inflammation may contribute to FAO development (2), which may be due to glucocorticosteroid insensitivity or resistance.

Impaired lung function and neutrophilic inflammation can persist despite corticosteroid treatment reducing eosinophilic inflammation (419), suggesting an alternate non-eosinophilic inflammatory pathway may play a role. The variable response to ICS may be due to the presence of inflammatory pathways, other than TH2 and eosinophilic inflammatory pathways, which has been demonstrated in severe asthma phenotypes (237). Furthermore airway neutrophilia has been associated with FAO (33), older people with asthma
(28) and severe asthma (206, 238, 420), suggesting airway neutrophilia reflects either steroid resistant and/or more severe disease. On the other hand, neutrophils may play a protective role because steroids inhibit neutrophil cell apoptosis (233, 234), which may contribute to neutrophil activation and increased neutrophil numbers.

As discussed in the previous chapter, TH_{17} inflammation may have a role in FAO because of its association with neutrophilic inflammation (244, 246, 421) and pro-inflammatory role as demonstrated by the association with increased proteolytic enzymes (245, 246).

Transcriptomics and gene analysis have been used in attempt to identify underlying mechanisms leading to FAO (422). A recent study using Gene Set Variation Analysis to assess sputum, nasal and endobronchial brushings and biopsies has shown that patients with severe asthma and FAO exhibit different gene expression profiles compared to those without FAO (422). Identified pathways involved IL-13 associated disease pathways including eosinophilic inflammation and remodeling involving impaired IFN-a function (422), which could be potential treatment targets.
1.9. Asthma-COPD Overlap
Asthma and COPD are usually regarded as separate disease entities (423) however both diseases are now recognized as being highly heterogeneous and can overlap. The term Asthma-COPD overlap (syndrome) or ACO(S) has been used to define this condition where features of both asthma and COPD are present (1, 424).

ACO has been reported to have a prevalence of about 20% in patients with obstructive airway disease and 2% in the general population (424-428). Patients with ACO have poorer outcomes including more frequent acute exacerbations and hospitalizations, health status impairment and increased mortality (425, 426, 428, 429). Co-morbidities are common in both diseases separately (COPD, severe asthma and asthma in the elderly) and therefore also contribute to significant disease burden (430, 431). The impact and significance of co-morbidities in ACO needs to be explored further as there may (425, 432, 433) or may not (428, 434) be an association with increased co-morbidities.

ACO may originate during childhood as long-standing childhood asthma may develop into incompletely reversible or FAO in adulthood (377, 435). The risk is higher in children with severe asthma (435) and in those who smoke (377). The association with childhood asthma and smoking leading to ACO has also been demonstrated using CT scans (436). This study used CT scans to measure airway dimensions and showed that in adult smokers with a history of childhood asthma, the airways were smaller compared smokers without childhood asthma (436). Other factors in childhood that may influence the risk
of ACO developing in adulthood include maternal factors during pregnancy such as smoking (437), atopy and infection (424). Factors that may affect lung growth during childhood such as premature birth, infection and genetic susceptibility also have to potential to contribute to ACO development later in life (438, 439).

The underlying mechanisms of ACO developing from childhood asthma are unknown. Unsurprisingly airway inflammation and airway remodelling are thought to contribute, as these processes also occur in childhood asthma (437). However, it is not known whether this contributes to incomplete lung growth and therefore development of ACO. Other factors demonstrated in a severe childhood asthma cohort (440) such as high exhaled nitric oxide, high healthcare use and high daily ICS dose, were identified as a cluster with ‘early-onset atopic asthma with advanced airflow limitation’. The authors proposed that these features, which are suggestive of severe eosinophilic childhood asthma, could lead to incompletely reversible airflow obstruction (440). A similar cluster was found in another study and this group of children responded the best to ICS/LABA therapy (441). However, the role of eosinophilic inflammation and ICS use in childhood asthma leading to ACO is questionable because another longitudinal study showed that adults with the lowest lung function had used ICS in childhood (371).

It is widely accepted that asthma is typically associated with eosinophilic and TH₂ driven inflammation (62) and in COPD neutrophilic inflammation dominates (442). However there is there is also heterogeneity of airway inflammation within and between the two diseases, with the presence of
eosinophilic and neutrophilic inflammation seen in both (443, 444). The pattern of airway inflammation in ACO has not been studied extensively. However, a large cross-sectional study of >4000 patients with airway disease, with 9% of the participants diagnosed with ACO (445), showed highly variable airway inflammatory profiles within this group (i.e. eosinophilic, neutrophilic and mixed) in keeping with the concept of ACO being a heterogeneous disease. The role of systemic inflammation in ACO is also unknown although systemic inflammation in ACO is similar to that seen in COPD (425, 446, 447).

Clinical practice guidelines regarding diagnosis, treatment and management of ACO are limited because evidence to date is scarce. Recommendations by expert opinion consensus (1) proposes diagnosing ACO based on the presence of typical features associated with either COPD or asthma. Treatment is then tailored towards the disease with the predominant features (1). FAO in asthma may fall under the category of ACO however there is no clear definition for either phenotype, further adding to the challenge of managing these conditions. The debate around ACO which has been in existence for years when the ‘Dutch hypothesis’ was presented in 1961 (448), seems to be more conflicting with the introduction of more physiological and molecular biomarkers. There are many unanswered questions regarding both phenotypes and ongoing research is essential to help characterize patients and guide treatment decisions for both FAO in asthma and ACO.
1.10. Summary
FAO in asthma is important because of the associated poor outcomes. Airway remodelling is thought to be the underlying process, with the small airways being the main site of pathology and inflammation being the driving factor. The contribution of lung tissue and the lungs elastic properties to airflow obstruction in asthma is under-recognized and likely plays a significant role, particularly in older people because of the changes that occur with ageing. Simple non-invasive tools to assess the contribution of the small airways and the lungs elastic properties are somewhat limited. Furthermore, the underlying mechanisms and physiological basis for FAO in older non-smokers with asthma remains largely unknown.

1.10.1. Aims
Therefore the aims of this thesis were to:

1. Assess a method of measuring small airway function using modern and currently available commercial devices.

2. Measure and quantify the lungs elastic properties in older non-smokers with asthma and FAO.

3. Determine the relationship between small airway function and the lungs elastic properties in this cohort.

4. Assess airway inflammation and determine its contribution to lung function impairment in this cohort.
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Chapter 2

2  *In vitro* and *in vivo* functional residual capacity and *in vivo* ventilation heterogeneity measurement comparisons between multiple breath nitrogen washout devices.
2.1 Introduction
Abnormalities in the small airways can be detected using the multiple breath nitrogen washout (MBNW) test, which provides a more sensitive measure of small airway function compared to spirometry (1-3). MBNW provides insightful information about the small airways, using indices that provide mechanistic information about ventilation heterogeneity (VH) in the conductive and diffusion dependent airways (Scond and Sacin) and assess global gas-mixing efficiency in the lung (Lung Clearance Index, LCI). These indices rely on accurate functional residual capacity (FRC) measurement (4).

Advances in technology have allowed the development of new commercial MBNW devices, however there are still limited published data comparing measurements between devices. Previous studies have compared in-house and/or commercial devices against standard body plethysmography as well as mass spectrometry, often considered the gold standard (5-8). Many of these studies have used lung models, assessed measurements in the paediatric population and used inert gases rather than nitrogen (N₂) (9-11). Limited published data have also shown inconsistencies between lung model and adult measurements (6).

Furthermore, the effect of software upgrades in a rapidly evolving field has not been extensively studied. Previous studies in one specific device have shown software changes can have a significant impact on results (12, 13). Recommendations for MBNW techniques have been published in the European Respiratory Society/American Thoracic Society (ERS/ATS) Consensus statement (4) yet on-going work is still needed to improve
standardisation of equipment, technical specifications and algorithms for calculation of its indices.

The aim of this study was to evaluate FRC and VH indices (Scond, Sacin and LCI) measured using two commercial MBNW devices: Exhalyzer® D (“EM”), ECO MEDICS AG, Duernten, Switzerland and EasyOne Pro® LAB (“ndd”), ndd Medical Technologies, Zurich, Switzerland. We also examined a third, independent, previously published (2) in-house device at the Woolcock Institute of Medical Research (“WIMR”), which measures N₂ directly in contrast to the commercial devices. FRC from the devices were compared against a known volume using a syringe lung model (in vitro) and against plethysmographic lung volume in healthy and asthmatic adult subjects (in vivo). In addition, we compared FRC analyses using two different software versions of the ndd device as a software update involving major changes to N₂ calculation had recently become available; previous data (14, 15) has shown significant FRC underestimation using the older, widely available software. VH indices were compared between the three devices in healthy and asthmatic adult subjects (in vivo).

2.2 Methods

2.2.1 Study design

In vitro and in vivo measurements were performed using the three different MBNW devices, in random order as determined by a computer generated randomisation sequence. The study was approved by the Northern Sydney Local Health District Human Research Ethics Committee (protocol number
LNR/16/HAWKE/11). Written informed consent was obtained from all recruited participants.

2.2.2 The lung model

In vitro measurements were performed using an optimised syringe lung model (Figure 2.1). A 3-litre (L) volume calibration Hans Rudolph® syringe (5530 Series) was modified to produce the physiological expirogram encountered during in vivo testing. This was accomplished by incorporating an attachment on the front of the syringe consisting of 18 flexible Tygon® (S3 E-3603) tubes of varying lengths (internal diameter of 0.048 cm and lengths ranging from 10-49.5 cm, permeability coefficients: \( \text{CO}_2=360\times10^{-11} \), \( \text{N}_2=40\times10^{-11} \) and \( \text{O}_2=80\times10^{-11} \) cm\(^2\).s\(^{-1}\).cmHg\(^{-1}\)), to produce the phase I and II portions of the expirogram, and a 3D-printed helical mixer device inserted at the syringe entrance to optimise gas mixing and produce a smooth phase III.

The target FRC was calculated by adding the known syringe volume to the dead space of the lung model attachments. The syringe volume was adjusted via a stopper on the syringe plunger to pre-determined positions. The dead space of the attachments was 0.310 L, determined from the Computer-Aided Design specifications and confirmed with water displacement.
Figure 2.1

Physical lung model comprised of (a) 3-L Hans Rudolph® syringe, (b) an attachment made from 18 flexible tubes, (c) a helical mixer device inserted at the syringe entrance and (d) the adjustable syringe stopper.
2.2.4 In vitro study

*In vitro* measurements were performed in triplicate on each device using four different FRC volumes (1.51 L, 1.81 L, 2.11 L and 2.31 L). A standardised adult protocol (tidal volume of 1-1.3 litres) (2, 4, 16-18) was used. After at least 5 syringe strokes with a stable end expiratory volume, the washout phase was commenced and 100% oxygen was switched on. Syringe strokes were continued until end-tidal N₂ concentration decreased to 1/40\(^{th}\) of the starting end-tidal N₂ concentration (4). Between each measurement, at least 10 strokes were performed prior to expel any residual oxygen within the syringe lung model and to ensure N₂ had returned to baseline. A single operator (KOT) performed all measurements under Ambient Temperature and Pressure Dry (ATPD) conditions.

2.2.5 In vivo study

Healthy volunteers were recruited from the WIMR and defined as current non-smokers with <10 pack-years smoking history and with no history of acute respiratory illness within the preceding month. Subjects with asthma were recruited if they had a physician diagnosis of asthma; history of asthma symptoms, previously documented significant bronchodilator reversibility on spirometry and/or a positive bronchoprovocation challenge test and on inhaled bronchodilator and/or inhaled corticosteroid medication. Short acting β-agonists were withheld for 6 hours and long-acting β-agonists for 24 hours before testing.

All participants were over the age of 18 years and completed a standardised interview on respiratory and general health prior to performing pulmonary
function tests (PFTs) in the following order: MBNW, spirometry and body plethysmography. All tests were performed in a single session at the Woolcock Institute of Medical Research.

Spirometry and body plethysmography were performed according to ATS/ERS Guidelines, using a Medisoft BodyBox 5500 (Medisoft Corporation, Sorrines, Belgium) (19, 20).

MBNW was performed as previously described (17). Tests were performed in triplicate in the seated position, using a nose clip and device-specific bacterial filter and mouthpiece attachments. Tests were conducted according to ERS/ATS consensus statement guidelines (4), using a standardised adult protocol (tidal volume of 1-1.3 litres) (2, 16-18). After at least 5 breaths with a stable end expiratory lung volume, the washout phase was commenced and 100% oxygen was switched on. Breaths were continued until end-tidal N₂ concentration decreased to 1/40th of the starting end-tidal N₂ concentration (4). The time interval between measurements was standardised to twice the previous washout time (4).

2.2.6 MBNW hardware and software

Details of the WIMR, EM, and ndd devices can be found in the appendix. Briefly, the WIMR device measures N₂ directly via a side-stream N₂ analyser (2). The commercial devices both measure N₂ indirectly, based on side-stream CO₂ and O₂ in the EM device and molar mass and CO₂ in the ndd device (6). Daily calibration and/or verification of each device were performed as described in the appendix.
FRC was calculated as the net volume of $N_2$ expired divided by the difference between end tidal $N_2$ concentration at the start and end of the washout portion of the test (4). FRC values were corrected for the pre-capillary dead space volume for each device. Lung volume turn over values (TO) were calculated for each breath over the washout as the cumulative expired volume (CEV), the sum of all tidal breaths over the washout, divided by FRC (21). LCI is a volume ratio and defined as the number of TO required to reduce alveolar $N_2$ concentration to $1/40^{th}$ of the starting concentration (i.e. CEV/FRC) (4).

The phase III slope of each breath was determined by linear regression from 50-95% of the exhaled volume of air, with manual adjustments performed to maximize inclusion of phase III and exclude contributions from phase II and IV (4). Each breath of all three MBNW test was analysed individually, whereby the alveolar phase III slope of nitrogen concentration versus expired volume of each breath, was divided by the corresponding mean nitrogen concentration (normalised slope, $S_n$). The normalised slopes were then plotted against lung turnover (CEV/FRC) and Scond was calculated as the linear regression slope between turnovers 1.5 and 6. Sacin was derived from the normalised slope of the first breath minus the contribution of the conductive airway ventilation heterogeneity (Mean $S_n$ of first breath – Scond x mean lung turnover of 1st breath) (17). Methods of $N_2$ estimation and Scond and Sacin calculation were according to device specific software.

MBNW data were analysed using software specific to each device. For the WIMR device custom-written software (Solver Version 1.3.2.18) was used. For the EM device Spiroware Version 3.1.6 was used. For the ndd device, the
same measurements were analysed using two different software versions and described in the appendix: clinical software Version 2.00.01.05 (termed “ndd v2.00”), and the recent upgrade released in March 2016, Version 2.01.00.09 (termed “ndd v2.01”). The ndd v2.01 software contains updates to both the N₂ calculation method and improved flow-gas delay synchronisation. Scond, Sacin and LCI were only analysed using ndd v2.00.

Measurements were included for analyses if acceptability criteria were met. For the in vitro study, measurements were deemed acceptable if three tests were within 10% of the mean FRC (20) value across the triplicate measures. For the in vivo study, measurements were deemed acceptable if two or more tests were performed adequately according to the ERS/ATS Consensus statement (4). Tests were excluded if there was evidence of leak during testing, tidal volume of the first breath or more than a third of breaths during washout was outside 1-1.3 L, and if the end-tidal N₂ concentration did not reach the recommended 1/40th of initial concentration during data acquisition.

2.2.7 Statistical analysis

Data were analysed using IBM SPSS Version 24 and graphs generated using GraphPad Prism Version 6.0. Summary data are presented as mean ±standard deviation (SD) for normally distributed data and median (inter-quartile range, IQR) for non-normally distributed data. Accuracy of in vitro FRC was assessed as per the consensus guidelines (4) (FRC values within 5% of known volume for at least 95% of values) and expressed as absolute (L) difference (measured FRC – lung model FRC) and relative (%) difference (absolute difference x 100/lung model FRC). In vivo FRC was compared with
body plethysmography FRC (FRC\textsubscript{pleth}). FRC differences between devices were assessed using Bland and Altman plots with 95% limits of agreement and by non-parametric one-way repeated measures ANOVA (Friedman’s test) and post-hoc tests. In vivo Scond, Sacin and LCI were compared separately in healthy and asthmatic subjects between the three devices and analysed using one-way repeated measures ANOVA. P-values <0.05 were considered statistically significant.

2.3 Results

2.3.1 *In vitro* FRC comparison

A total of 108 measurements were performed across the three MBNW devices (Table 2.1). Differences between measured and lung model FRC are shown in Figure 2.2 and Table 2.2. FRC accuracy was within the specified 5% accuracy range of the lung model FRC for 100% of WIMR measurements and 97% of EM measurements. All FRC measurements using ndd v2.00 were underestimated and not within the specified 5% accuracy range. Using ndd v2.01, accuracy improved though only 36% of measurements were within the 5% accuracy range.
Table 2.1 In vitro FRC (in litres) measurements on each device.

<table>
<thead>
<tr>
<th>Lung model FRC volume</th>
<th>n</th>
<th>WIMR</th>
<th>EM</th>
<th>ndd v2.00</th>
<th>ndd v2.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.31</td>
<td>9</td>
<td>2.30 ± 0.02</td>
<td>2.39 ± 0.02</td>
<td>1.78 ± 0.14</td>
<td>2.16 ± 0.04</td>
</tr>
<tr>
<td>2.11</td>
<td>9</td>
<td>2.10 ± 0.02</td>
<td>2.17 ± 0.02</td>
<td>1.72 ± 0.12</td>
<td>2.00 ± 0.03</td>
</tr>
<tr>
<td>1.81</td>
<td>9</td>
<td>1.84 ± 0.02</td>
<td>1.88 ± 0.02</td>
<td>1.37 ± 0.11</td>
<td>1.72 ± 0.03</td>
</tr>
<tr>
<td>1.51</td>
<td>9</td>
<td>1.53 ± 0.03</td>
<td>1.53 ± 0.02</td>
<td>1.25 ± 0.05</td>
<td>1.43 ± 0.02</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD. FRC: functional residual capacity, WIMR: Woolcock in-house device, EM: Exhalyzer® D device, ndd v2.00: EasyOne Pro® LAB device Version 2.00.01.05, ndd v2.01: EasyOne Pro® LAB device analysed in Version 2.01.00.09.
Figure 2.2
Bland Altman plots of in vitro functional residual capacity (FRC) measurements on each device.

Data plotted as measured FRC minus lung model FRC, expressed as the absolute difference vs mean of measured and lung model FRC. Absolute differences (closed circles), mean difference and upper and lower limits of agreement (mean difference ±SD of differences) are shown as dotted lines.

WIMR: Woolcock in-house device, EM: Exhalyzer® D device, ndd: EasyOne Pro® LAB device analysed in Version 2.01.00.09.
Table 2.2 Differences in vitro FRC measurements from the lung model FRC for 4 different lung volumes.

<table>
<thead>
<tr>
<th>Device</th>
<th>WIMR</th>
<th>EM</th>
<th>ndd v2.00</th>
<th>ndd v2.01</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Absolute</td>
<td>%</td>
<td>Absolute</td>
</tr>
<tr>
<td>Lung model FRC volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.31</td>
<td>9</td>
<td>-0.01 ± 0.02</td>
<td>-0.2 ± 0.8</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>2.11</td>
<td>9</td>
<td>-0.01 ± 0.02</td>
<td>-0.43 ± 0.9</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>1.81</td>
<td>9</td>
<td>0.03 ± 0.02</td>
<td>1.59 ± 1.35</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>1.51</td>
<td>9</td>
<td>0.02 ± 0.03</td>
<td>1.68 ± 13.1</td>
<td>0.02 ± 0.02</td>
</tr>
</tbody>
</table>

Data presented as mean absolute difference (Absolute, L) and mean percentage difference (%) from the lung model FRC. FRC: functional residual capacity, WIMR: Woolcock in-house device, EM: Exhalyzer® D device, ndd v2.00: EasyOne Pro® LAB device Version 2.00.01.05, ndd v2.01: EasyOne Pro® LAB device analysed in Version 2.01.00.09.
2.3.2 *In vivo FRC* comparison

Twenty-nine subjects (20 healthy controls and 9 asthmatics) were included in the analyses and their characteristics are outlined in Table 2.3. Mean (±SD) FRC measured by the WIMR (3.27±0.82 L) and EM (3.56±0.92 L) devices did not differ significantly from FRC_{pleth} (3.44±0.77 L) or from each other. FRC measured by ndd v2.00 (2.71±0.64 L) was significantly lower than FRC_{pleth}, WIMR and EM (p<0.0001) (Figure 2.3 and Table 2.4). The same pattern of FRC differences was observed in healthy control and asthmatic subjects.

When the ndd data was re-analysed using ndd v2.01 software, end tidal N\textsubscript{2} concentrations became systematically higher, such that only 9 of the 29 subjects had measurements that reached an acceptable end-tidal N\textsubscript{2} concentration by the end of the washout. However a majority of the measurements exhibited a stable plateau at the end of the washout. For the purposes of this study, all 29 subjects were included for analysis to allow comparison of the effect on FRC. FRC increased when re-analysed using ndd v2.01 (3.06±0.71 L) and was still significantly lower than FRC_{pleth} (p=0.011) and the EM device (p=<0.0001); however did not differ from the WIMR device.
Table 2.3 Subject demographics and standard lung function measurements.

<table>
<thead>
<tr>
<th></th>
<th>All subjects n=29</th>
<th>Controls n=20</th>
<th>Asthmatics n=9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M:F</strong></td>
<td>12:17</td>
<td>8:12</td>
<td>4:5</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>37 ± 12</td>
<td>36 ± 12</td>
<td>37 ± 13</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>172 ± 12</td>
<td>174 ± 12.5</td>
<td>168 ± 10</td>
</tr>
<tr>
<td><strong>BMI (kg.m (^2))</strong></td>
<td>24.1 ± 3.63</td>
<td>23.9 ± 3.48</td>
<td>24.6 ± 4.10</td>
</tr>
<tr>
<td><strong>Smoking history (pack years)</strong></td>
<td>2.5 ± 3.2</td>
<td>2.75 ± 3.31</td>
<td>0.70 ± 0.00</td>
</tr>
<tr>
<td><strong>FEV(_1) (L)</strong></td>
<td>3.66 ± 0.93</td>
<td>3.75 ± 0.93</td>
<td>3.47 ± 0.95</td>
</tr>
<tr>
<td><strong>FVC (L)</strong></td>
<td>4.75 ± 1.17</td>
<td>4.77 ± 1.16</td>
<td>4.71 ± 1.25</td>
</tr>
<tr>
<td><strong>FEV(_1)/FVC</strong></td>
<td>0.77 ± 0.06</td>
<td>0.79 ± 0.05</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td><strong>TLC (L)</strong></td>
<td>6.51 ± 1.38</td>
<td>6.55 ± 1.41</td>
<td>6.42 ± 1.41</td>
</tr>
<tr>
<td><strong>FRC(_{pleth}) (L)</strong></td>
<td>3.44 ± 0.77</td>
<td>3.49 ± 0.78</td>
<td>3.34 ± 0.80</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD. BMI: body mass index, FEV\(_1\): forced expiratory volume in 1 second, FVC: forced vital capacity, TLC: total lung capacity, FRC\(_{pleth}\): functional residual capacity measured using body plethysmography.
Figure 2.3
Bland Altman plots of in vivo functional residual capacity (FRC) measurements on each device.

Data plotted as body plethysmography FRC (pleth) minus multiple breath nitrogen washout (MBNW) FRC, expressed as the absolute difference vs mean of pleth and MBNW FRC. Absolute differences (closed circles), mean difference and upper and lower limits of agreement (mean difference ±SD of differences) are shown as dotted lines. WIMR: Woolcock in-house device, EM: Exhalyzer® D device, ndd: EasyOne Pro® LAB device analysed in Version 2.01.00.09.
Table 2.4 Differences in in vivo FRC measurements from body plethysmography FRC on each device.

<table>
<thead>
<tr>
<th>Device</th>
<th>WIMR</th>
<th>EM</th>
<th>ndd v2.00</th>
<th>ndd v2.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Absolute</td>
<td>Absolute</td>
<td>Absolute</td>
<td>Absolute</td>
</tr>
<tr>
<td>Controls</td>
<td>-0.13 ± -3.3</td>
<td>0.17 ± 4.7</td>
<td>-0.73 ± -20.6</td>
<td>-0.36 ± -10.1</td>
</tr>
<tr>
<td></td>
<td>0.46 § ± 13.0</td>
<td>0.41 ± 11.8</td>
<td>0.36 ± 8.9</td>
<td>0.32 ± 8.4</td>
</tr>
<tr>
<td>Asthmatics</td>
<td>-0.24 ± -6.8</td>
<td>0.01 ± 0.12</td>
<td>-0.73 ± -20.7</td>
<td>-0.42 ± -11.2</td>
</tr>
<tr>
<td></td>
<td>0.45 ± 13.8</td>
<td>0.39 ± 12.0</td>
<td>0.59 ± 15.3</td>
<td>0.57 ± 15.8</td>
</tr>
<tr>
<td>All subjects</td>
<td>-0.17 ± -4.4</td>
<td>0.12 ± 3.3</td>
<td>-0.73 ± -20.6</td>
<td>-0.38 ± -10.5</td>
</tr>
<tr>
<td></td>
<td>0.45 ± 13.1</td>
<td>0.40 ± 11.8</td>
<td>0.43 ± 11.8</td>
<td>0.40 ± 10.9</td>
</tr>
</tbody>
</table>

Data presented as mean absolute difference (Absolute, L) and mean percentage difference (%) from body plethysmography FRC. FRC: functional residual capacity, WIMR: Woolcock in-house device, EM: Exhalyzer® D device, ndd v2.00: EasyOne Pro® LAB device Version 2.00.01.05, ndd v2.01: EasyOne Pro® LAB device analysed in Version 2.01.00.09. § Missing data for 1 subject.
2.3.4 *In vivo* Scond, Sacin and LCI comparisons: between devices in healthy control and asthma subjects

*In vivo* VH indices measured in healthy and asthma subjects using each device are outlined in figure 2.4. Due to less than a third of the subjects not meeting acceptability criteria when the ndd data was reanalysed using ndd v2.01 software, only ndd v2.00 data was used for these comparisons.

In the healthy subjects Scond was comparable across all three devices (median (IQR) Scond: WIMR 0.011 (0.009-0.022) L⁻¹, EM 0.015 (0.013-0.023) L⁻¹ and ndd v2.00 0.014 (0.008-0.020) L⁻¹. In the asthmatic group, Scond measured by the WIMR (0.020 (0.014-0.023) L⁻¹) and EM (0.025 (0.019-0.032) L⁻¹) devices were both comparable to ndd v2.00 (0.021 (0.013-0.027) L⁻¹). However, Scond on the WIMR device was significantly lower compared to the EM device (p=0.03) but the differences were small.

Sacin in the healthy subjects was comparable between the WIMR (0.095 (0.069-0.119) L⁻¹) and ndd v2.00 (0.100 (0.080-0.129) L⁻¹) devices, yet significantly lower on the EM device (0.059 (0.050-0.077) L⁻¹; p=0.03 and p<0.0001 for comparisons with WIMR and ndd v2.00, respectively. In the asthmatic subjects, Sacin was similar between the WIMR (0.082 (0.059-0.131) L⁻¹) and EM (0.066 (0.050-0.095) L⁻¹) and ndd v2.00 devices (0.114 (0.070-0.137) L⁻¹). However Sacin was significantly lower on the EM compared to the ndd v2.00 device (p=0.003).

LCI in the healthy subjects was significantly lower on the WIMR (6.93 (6.48-7.11)) compared to the EM (7.18 (6.89-7.57); p<0.0001) and ndd v2.00 devices (7.18 (6.64-7.63); p<0.0001), and comparable between the EM and
ndd v2.00 devices. In the asthmatic subjects, LCI was comparable between all three devices (WIMR: 6.81 (6.69-7.38), EM: 7.30 (6.72-7.62), ndd v.200: 6.92 (6.46-7.54)). Although LCI was comparable in the asthmatic group, the difference in the raw median values were more variable compared to the healthy controls and likely related to small numbers.
Figure 2.4

In vivo Scond, Sacin and LCI measurements on each device in healthy (top panel) and asthma subjects (bottom panel).

Data presented as median (inter-quartile range). Scond: ventilation heterogeneity in conductive-dependent airways, Sacin: ventilation heterogeneity in diffusion-dependent airways, LCI: lung clearance index, WIMR: Woolcock in-house device, EM: Exhalyzer® D device, ndd: EasyOne Pro® LAB device Version 2.00.01.05.
2.4 Discussion

2.4.1 Summary of results

This is the first study to compare *in vitro* and *in vivo* FRC measurements and *in vivo* VH indices in healthy and asthmatic adults, between two currently available commercial and one in-house MBNW device. The WIMR device measured *in vitro* FRC closest to the known lung model volume. Mean overestimation of *in vitro* FRC and FRC_{pleth} was 3% by the EM device, in comparison to a mean 21% underestimation by the ndd device. However on re-analysis using ndd v2.01 underestimation was minimised to 5% (*in vitro*) and 11% (*in vivo*), respectively. There were statistically significant differences in FRC measurements between commercial MBNW devices although this difference was relatively small between EM and ndd v2.01. Furthermore, the pattern of differences (i.e. over-estimation or under-estimation of FRC) between devices was consistent using both the *in vitro* physical lung model and in *in vivo* measurements.

*In vivo* VH measurements in were variable across the three devices in both the healthy control and asthma subjects, although with some indices the differences were small. In each group, Scond measured highest using the EM device, followed by the ndd v2.00 then WIMR devices. Scacin was highest using the ndd v2.00, followed by the WIMR and EM devices. LCI on the other hand did not follow a similar pattern to Scond or Scacin measurements in either group. The pattern of *in vivo* differences in Scond, Scacin and LCI across the devices did not follow the same pattern of differences seen with *in vitro* and *in vivo* FRC measurements.
2.4.2 Comparison to other studies

The Consensus recommendations based on expert opinion stated that 95% of the *in vitro* FRC measurements should be within 5% of the target volume (4). WIMR and EM devices fulfilled this criterion, however the ndd v2.00 device did not achieve 5% accuracy for any measurements. When measurements were re-analysed using ndd v2.01, 36% fulfilled this criterion. Both commercial devices were reported previously to be highly accurate in measuring FRC using a physical lung model (14). This lung model was water-based and incorporated BTPS (body temperature, ambient pressure, saturated) correction whereas ours did not. Despite this, our results showed the same pattern of over/underestimation *in vitro* and *in vivo*. This is consistent with their *in vivo* results in 10 healthy adults, where EM overestimated FRC\textsubscript{pleth} by 14% and ndd underestimated by 23% (14). A recent study with healthy control and cystic fibrosis subjects also reported the same pattern, i.e. higher FRC measurements using the EM device compared to the ndd device (15). Other *in vitro* and *in vivo* paediatric studies exist (9, 11), however they used different tracer gases, SF\textsubscript{6} and helium, which limits comparison.

Only one other study has compared LCI between the EM and ndd devices in both children and young adults using healthy controls and subjects with cystic fibrosis (15). LCI measured using the EM device was significantly higher than the ndd in all age groups and in both health and disease (15). In contrast, our results showed LCI was similar between both commercial devices in both the healthy and asthma subjects. Poncin *et al* as expected, also showed LCI to be
higher in disease compared to healthy controls, regardless of the device. In our study, LCI was higher in those with asthma compared to healthy control subjects but only using the EM and not the ndd device. Different ndd software versions, differences in FRC measurements and smaller numbers in our study may explain these observed differences.

Other paediatric (9) and adult (8) studies have compared LCI using different wash out devices and tracer gases. Differences in LCI were observed in the paediatric study, which compared measurements between the mass spectrometer and EM devices (9). The mass spectrometer device used SF$_6$ as the tracer gas, whereas the EM device used O$_2$. In this study, the EM device also measured LCI higher compared to the mass spectrometer, in both healthy control and cystic fibrosis paediatric subjects (9). This was thought to be related to the greater number of breaths required to complete the washout on the EM compared to the mass spectrometer device (9). The number of breaths required to complete the washout on the EM device also increased proportionally to the volume of trapped gas, which makes sense because the EM device also measured FRC higher than the mass spectrometer. Unsurprisingly, LCI was significantly higher in those with cystic fibrosis compared to healthy controls, regardless of the device (9). On the other hand, the study in healthy adults using small numbers showed comparable LCI values between two other devices, the mass spectrometer and an ultrasonic flow sensor prototype system, despite statistically but not clinically significantly differences in FRC (8). The use of different devices and tracer gases in these studies limits comparisons to ours.
To our knowledge there have been no other studies comparing Scond and Sacin between different MBNW devices. Furthermore, there is no gold standard MBNW device to compare our Scond and Sacin results to.

2.4.3 Impact of N₂ estimation method and Scond and Sacin calculation method

Differences in FRC across the three devices are more likely attributed to device and software variations. This includes the method used to calculate N₂ concentration. The WIMR device measures N₂ directly, while the EM device uses Dalton’s law to compute N₂ by simple subtraction of other constituent gases in the expired air; these two methods were found to be similar. In contrast, the ndd v2.00 device uses the concept of a prototype expirogram, derived from the shape of the molar mass versus expired volume curve in the early breaths of the washout. The expired N₂ volume for each breath is then determined by scaling the prototype expirogram to match the end-expiratory N₂ concentration for that breath. Potential inaccuracies could occur if expirogram shape changed greatly during the course of the washout, as typically observed in more severe obstructive airways disease. Ndd v2.01, however, used a combination of molar mass measurements as well as Dalton’s law to compute N₂, on a point-by-point basis for the entire expirogram. This could partly explain the improved accuracy of ndd v2.01 and reduced FRC discrepancy between the new software and the WIMR and EM devices.

VH indices rely on FRC accuracy; therefore variation in FRC is likely to impact these. Differences in LCI between devices would be expected to mirror the
differences seen in FRC measurements because LCI is a volume ratio determined by the number of lung turnovers (TO = CEV/FRC). An increase in FRC should therefore result in an increase in LCI, which was observed in our study with the EM device and other studies (9, 15). Additionally, the same trend may be expected with Scond, as Scond incorporates normalised phase III slopes (therefore N₂ concentration) and lung turnovers. However, our results have not shown Scond differences to follow the same pattern as FRC.

The ndd device underestimated FRC, yet Scond measurements were between that of the EM and WIMR devices, in both healthy and asthmatic subjects. Sacin on the other hand may not necessarily be affected by FRC, as it takes into account the first breath of the wash out. On the other hand, the calculation of Sacin also incorporates Scond therefore may be affected by variations in FRC, however there is no evidence to confirm or refute this. In our study, the pattern of differences in Sacin did not follow the same trend seen in FRC measurements and in fact was in the opposite direction.

We can speculate that the variability in VH indices between the devices may be due to different methods of N₂ estimation, as each device and their respective software calculate Scond and Sacin using different methods. However, this is unlikely to be the only explanation for the observed inter-device differences because unpublished data from our lab has demonstrated comparable Scond and Sacin results using the three different calculation methods employed by each of the devices in this study. This analysis was however only performed on measurements from one device (EM) in 39 healthy subjects and the effect disease may have is unknown. Other possible
factors contributing to variability in Scond and Sacin include analysis of the expirogram and phase III slope. The WIMR and EM devices allow manual adjustment of the phase III slopes whereas the ndd device does not. One person performed all the analyses and minimal slope adjustments were made, only as deemed necessary according to the consensus statement (4). A previous study in children with cystic fibrosis and healthy controls also showed automated Scond analysis and calculation was comparable to manual analysis, using MBNW measurements from one device (EM) (22). Therefore the contribution from variations in phase III slope analysis may be negligible however this needs further evaluation using all the devices.

2.4.4 Impact of other software changes
The other major change in ndd v.2.01 involved modifications to the estimation of delay between the flow and respective gas measurement points, which are more robust to variation in breathing patterns particularly very brief pauses in flow (personal communication with the manufacturer); differences in delay time potentially have a large effect on FRC calculations (23, 24).

Newer software versions are currently available at the time of writing (v.2.2.0.15 onwards), which involve major changes to the user interface, but fundamentally employ the same indirect N₂-based FRC calculation and delay estimation method. Thus our results have significant implications on the reanalysis of old data and are relevant to ndd v2.01 as well as any newer versions. Furthermore, they illustrate the importance of analysis methods and any pre-processing algorithms that may be used, and the need for software
transparency (25). They also highlight the importance of ongoing validation, and that standardisation efforts are gradually working.

It should be noted this study was unable to confirm whether collecting data using ndd v2.01 would have further improved FRC accuracy for the ndd device. A large proportion of in vivo tests re-analysed using the new software failed to meet end of test criteria, possibly due to underestimation of N₂ in the old software. Due to this technical limitation further analysis of the VH indices using the updated ndd software was not performed. One could speculate that if acceptable end-tidal N₂ concentration had been met, in vitro FRC may have been slightly closer to the true lung volume and in vivo FRC closer to FRC\textsubscript{pleth}. Furthermore, if in vivo FRC was closer to FRC\textsubscript{pleth} then VH indices using the ndd device may have been more comparable to the other devices.

### 2.4.5 Impact of patient factors

Other factors that may affect FRC and VH measurements include the effect of breathing patterns during the washout. Different mouthpieces, resistances, the nature of the real-time displays of volume and patient breathing incentives, even different open bypass systems delivering oxygen (15) may have affected breathing patterns. However, dead space correction was applied in each device and the same 1L-breathing protocol was used. More importantly, the direction of over/underestimation was preserved regardless of in vitro and in vivo FRC comparisons, i.e. independent of BTPS correction, breathing pattern and presence of CO₂. Thus it is unlikely that patient factors contributed significantly to the differences between devices. In addition, the differences between MBNW FRC and FRC\textsubscript{pleth} on each device were of similar
magnitude between healthy controls and subjects with asthma with well-preserved spirometry. It is unknown whether the same results would hold in subjects with more airway obstruction, or other patient groups such as COPD.

It is difficult to know whether patient factors contributed to differences in VH indices because FRC was not comparable between all three devices. Median LCI values were higher in the healthy compared to asthmatic subjects using the WIMR and ndd devices. This does not make physiological sense and is not consistent with findings from other studies (9, 15). In addition, the inconsistencies in the pattern of differences in Scond and Sacin in healthy and asthmatic subjects between devices are odd. The small numbers in our study limits any definitive conclusions that can be drawn regarding the impact of patient factors in relation to disease.

2.4.6 Limitations

Our study had a number of limitations: Firstly, we compared differences between devices both in vitro and in vivo only using FRC, but only in vivo comparisons between Scond, Sacin and LCI were performed. There are however no known lung models to evaluate these other indices. Nevertheless, high quality FRC measurement is necessary for evaluation of these indices. Secondly, between-session variability for FRC has not been defined therefore it is not known whether differences between in vivo measurements are within the limits of normal test variability. Thirdly, the applicability of our results to the paediatric population is unknown. However the simplistic design of the lung model could be easily adapted to a smaller syringe. Also, we only evaluated the standardised 1-L breathing protocol (26) hence the effect of the free
breathing protocol used by other groups (21) and especially in infants and children is unknown. Furthermore, the syringe is an ATPD rather than BTPS model, which is less representative of actual physiological situation but more practical for routine laboratory use. Despite this, the same pattern of accuracy was seen in vitro and in vivo suggesting that incorporation of BTPS correction has a minimal effect on the relative errors reported. Finally, as discussed above we did not evaluate the accuracy of data collected using the ndd v2.01, which is scope for further work.

2.5 Conclusion

We have shown differences in the measurements of FRC between three MBNW devices in a physical syringe model of the lung. The syringe lung model used in this study was simple, portable, and relatively easy to produce compared to that used in other studies (6, 27, 28). It would allow a simple and practical way to calibrate or check the MBNW setup since it tests the combined accuracy of volume and N₂ concentration measurement during a more realistic expirogram and the correct alignment of these signals. The use of such a syringe model may be beneficial for comparison between devices and laboratories, and for quality assurance monitoring. FRC differences were also reflected in vivo in healthy and asthmatic subjects, in relation to plethysmographic FRC. While further work is required to improve accuracy, the in vivo differences observed are small and likely not clinically significant. Differences in VH indices were observed across the devices in healthy and asthmatic subjects. The differences were not consistent between the indices across the devices and the contribution from differences in FRC measurement
cannot be ignored. Differences are likely to reflect the method of calculating \( N_2 \) concentration and other software and phase III analysis factors. How these FRC errors translate to accuracy of other indices has been explored for LCI in other studies (9, 15), however ours is the first study to explore Scond and Sacin. Further studies to include systematic phase III slope analysis with larger numbers and different disease states are required to explore the variability in these indices. Nevertheless, our results show that the state of the art is closer towards better comparability and standardisation for FRC accuracy across existing MBNW devices.

**Acknowledgments**

The authors acknowledge the contribution of Gunnar Unger, Martin Turner, Aaron Skelsey and Sunny Ye who were involved with the development of the syringe lung model. Both manufacturers for the Eco Medics and ndd devices were consulted for technical accuracy of the manuscript. The authors acknowledge the contribution of Dr. Christian Buess, who provided additional technical information regarding the ndd software where indicated in the manuscript. Neither manufacturer influenced the study design, results or interpretation.


Chapter 3

3 Lung elastic recoil and ventilation heterogeneity of diffusion-dependent airways in older people with asthma and fixed airflow obstruction.
3.1 Introduction
The small airways are the site of airflow obstruction (1, 2) and are abnormal in asthma (3). As discussed in previous chapters, one measurement of small airway function is Sacin, derived from the multiple breath nitrogen washout (MBNW) test (4-6), and reflects ventilation heterogeneity (VH) in diffusion-dependent airways. Other measures derived from the MBNW test include Scond, which reflects VH in the convective-dependent airways and lung clearance index (LCI), which reflects overall VH.

In asthma Sacin is correlated with airway hyper-responsiveness (7), asthma control (8) and improves with ultrafine inhaled corticosteroid treatment (9). Theoretically, heterogeneity of diffusion-dependent ventilation can arise due to the heterogeneity of cross-sectional areas of airway openings in terminal airways and the acini (10). Therefore, Sacin may be affected by structural changes in those airways. The elastic properties of the lung may also affect Sacin, as the phase III slope, a marker of VH derived from the single breath nitrogen washout, correlates with lung compliance in explanted lungs of smokers and in healthy lungs (11). Scond, on the other hand, is thought to be affected by the mechanical properties of the conducting airways, that is the lung units larger than the acini (12). Therefore Scond may not be affected by structural changes in acini.

Reduced lung elastic recoil makes a large contribution to airflow obstruction in asthma (13, 14), particularly in older individuals with long-standing disease who may develop fixed airflow obstruction (FAO) (15-17). FAO typifies chronic obstructive pulmonary disease, but can occur in people that have never
smoked, despite adequate treatment (14, 15), and is associated with poor outcomes (16, 18).

Since loss of elastic recoil is associated with age (19, 20) and asthma (21, 22) and Sacin is more abnormal in older asthmatics compared to younger (7), we hypothesised that the increase in Sacin in older people with asthma was due to loss of lung elastic recoil. Therefore the primary aim of this study was to examine the relationships between Sacin and lung elastic recoil pressure and compliance. Secondary aims of this study were to (i) examine the relationship between other MBNW indices, Scond and LCI, and the lungs elastic properties and (ii) examine the relationship between MBNW indices and other clinical parameters: standard lung function, age of asthma onset and symptoms.

3.2 Methods
3.2.1 Subjects
Adult subjects were recruited from a volunteer database and from referrals by local respiratory physicians. Subjects were eligible if they were aged 40 years or older, as abnormalities in elastic recoil are increasingly common from middle age onwards (13). All subjects were non-smokers, had a physician-confirmed diagnosis of asthma based on typical symptoms, previous evidence of variable airflow obstruction and a history of asthma medication use. Exclusion criteria were a past smoking history of ≥5 pack-years, an asthma exacerbation within the past 8 weeks, the presence of significant cardiac disease or respiratory disease other than asthma. Written informed consent was obtained from all subjects. Ethics approval was granted by the Sydney
Local Health District Human Research Ethics Committee (#HRECT/14/CRGH/75).

3.2.2 Study design and lung function measurements

Eligible subjects were treated with maximal dose inhaled corticosteroid/long-acting β-agonist (ICS/LABA) using Fluticasone/Eformoterol 250µg/10µg metered dose inhaler via a holding chamber, two puffs twice daily for two months. The rationale was to optimize asthma control and to ensure that any abnormalities that were related to steroid-responsive inflammation would be eliminated by this standardised asthma treatment (23). If subjects developed an exacerbation during the study period, lung function testing was delayed for a minimum period of at least one month after clinical improvement. ICS/LABA and short acting beta-agonist medications were withheld for at least 24hr and 6hr prior to testing.

Subjects completed the 5-item Asthma Control Questionnaire (ACQ-5) (24) and performed standard lung function (spirometry, body plethysmography and diffusing capacity) (25-27) and MBNW (Exhalyzer® D, ECO MEDICS AG, Duernten, Switzerland) as described in the previous chapter, both during enrolment and after two months of treatment. Post-bronchodilator spirometry was performed after one month of treatment to assess for the presence of FAO. FAO was defined as a spirometric ratio less than the lower limit of normal (28) and/or no significant bronchodilator response post 400 mcg of inhaled Salbutamol (29). At the end of the two-month treatment period in a single session following standard lung function and MBNW measurements, the lungs elastic properties were assessed. MBNW indices (Sacin, Scond and
LCI) were derived as previously described (30). Predicted values were reported according to published reference equations (19, 28, 30-32).

3.2.2.1 Measurement of the lungs elastic properties
The elastic properties of the lung were measured, as previously described using simultaneous measurements of gas flow (volume) and intra-oesophageal pressure (33, 34). Gas flow at the mouth was measured with a differential pressure pneumotachograph. Change in lung volume was obtained by integration of the instantaneous flow signal (33). Intra-oesophageal pressure was measured with a ± 75 cmH₂O transducer (DC030NDC4) connected via a catheter with a distal 10 cm balloon (Marquat Genie Biomedical). The phase difference between pneumotachograph and the balloon catheter was measured at 0.2 seconds using a rapid syringe push in a physical model. Volume and pressure calibration were performed prior to testing each subject. A 3-L syringe was used to calibrate volume and a water-manometer was used to calibrate pressure.

The balloon catheter was inserted via the nose into the lower third of the oesophagus (figure 3.1), after applying topical lignocaine gel and spray to the nose and throat. Catheter placement was determined using a standard equation (33) and once in position was secured to the nose using surgical tape. The balloon was then inflated with 0.5 ml of air. Immediately prior to each measurement both the oesophageal pressure transducer and mouth pressure transducer were zeroed to atmospheric pressure and the flow transducer was zeroed to conditions of no airflow. During the measurement,
trans-pulmonary pressure \( (P_{st(L)}) \) was determined as the difference between mouth and balloon pressure and shown in real time on a monitor.

**Figure 3.1**

Adapted from Benditt et al (35).

Schematic of equipment required for recording pressure signals from an oesophageal balloon catheter.
Measurements were performed in duplicate in the seated position, using a nose clip and mouthpiece (SureGard L/F Filter (purple) RJVKB2). Stable tidal breathing was established then subjects performed three slow breaths to TLC. Subjects then inhaled again to TLC after which the airway opening was occluded (figure 3.2) Subjects were instructed to relax down to FRC against the closed mouthpiece. During the passive deflation to FRC, 2-3 second interruptions were performed to allow recording of stable pressure at each volume (figure 3.3). Static $P_{st(L)}$ and lung volume were measured during this interrupted deflation from TLC (33, 36). At least five deflation manoeuvres were obtained during one measurement. The measurement ended with another period of stable tidal breathing and three slow breaths to TLC.
Figure 3.2
Breathing protocol for measurement of the lungs elastic properties.

Figure 3.3
Volume and pressure recording during deflation manoeuvre (measured from total lung capacity (TLC) to functional residual capacity (FRC)).
Pressure-volume (P-V) curves were constructed from all acceptable points (at least 30-40 points) obtained during all acceptable deflation manoeuvres. Points obtained near TLC were also used to avoid bias by a disproportionate number of values in the lower volume range (33). Using the equation of Colebatch (33, 37), the following exponential function was fitted to the P-V curve data points using a least-squares method; \( V = A - Be^{KP} \), where \( V \) = volume as %TLC; \( P \) = transpulmonary pressure; and \( A, B \) and \( K \) are constants (figure 3.4). “A” is the horizontal asymptote of the Y-axis, “B” is the distance on the Y-axis between “A” and the extrapolated Y-axis intercept of the curve. Therefore, “B/A” is an index that describes lung compliance with low values indicating leftward shift of the PV curve or reduction in recoil pressure. “K” is a shape factor that is an index of elastic recoil and is directly related to alveolar size and lung distensibility (38) with a greater curvature (higher \( K \)) indicating increased lung compliance. Both a reduced \( B/A \) and increased \( K \) are indicative of a loss of lung elastic recoil (i.e.: increased lung compliance). “B/A” and “K” are independent of lung size and their predicted values were calculated using equations of Colebatch et al (19).

The exponential function was fitted to the P-V curves between 50 to 100% of TLC, with the TLC being referenced to the value obtained by body plethysmography. A lower limit of 50% was defined by Colebatch et al because the constants \( B/A \) and \( K \) are influenced by the range of data over which the exponential is fitted (33). Explicitly defining a lower limit for the exponential fit of the P-V curve also improved reproducibility of P-V curve analysis (33).
Figure 3.4

The pressure-volume (P-V) curve fitted with the Colebatch equation: \( V = A - B e^{-KP} \) (33). \( V \): volume as %TLC; \( P \): transpulmonary pressure; \( A \), \( B \) and \( K \) are constants. \( A \): horizontal asymptote of the Y-axis, \( B \): distance on the Y-axis between “A” and the extrapolated Y-axis intercept of the curve, \( K \): shape factor of the exponential analysis of the P-V curve and reflects lung compliance.
3.2.3 Statistical analyses
Data are presented as means ±SD for normally distributed data and as medians (interquartile range IQR) for non-normally distributed data. Spirometry, lung volumes, diffusing capacity, MBNW and lung elastic recoil data are expressed as raw values, z-scores and/or percent predicted. Differences between spirometry and MBNW during enrolment and at the end of the study period were compared using paired t-tests. Correlations between pre-bronchodilator MBNW and pressure-volume indices were assessed using Spearman’s rank test. P<0.05 was considered significant.

3.3 Results
A total of 21 subjects were enrolled and two were excluded because they were unable to complete the lung function tests. Elastic recoil measurements were performed in 18 of the remaining 19 subjects (11 male). Anthropometric and lung function data are presented in Table 3.1. All subjects were taking an ICS with or without LABA; five subjects were also taking a long-acting muscarinic antagonist. One subject was on long-term low dose oral corticosteroids (Prednisone dose 5 mg) for rheumatoid arthritis.

Subjects were older, with a mean ±SD age of 63 ±9 years and long duration of asthma (38.2 ±22 years). Five subjects were ex-smokers (pack year history 2.2 ±2.5 years). Subjects had mildly uncontrolled asthma symptoms (ACQ-5: 1.03 ±0.92). Post-bronchodilator (BD) spirometry after one month of treatment showed moderate FAO: mean ±SD z-score forced expiratory volume in 1 second (FEV₁) -2.1 ±0.7, forced vital capacity (FVC) -0.6 ±1.0 and FEV₁/FVC -2.5 ±0.9. After two months, baseline spirometry had not changed (pre-BD
FEV\textsubscript{1} -2.1 ±0.7, FVC -1.0 ±1.1 and FEV\textsubscript{1}/FVC -2.4 ±0.9; paired t-test: p=0.3, p=0.3 and p=0.6 for FEV\textsubscript{1}, FVC and FEV\textsubscript{1}/FVC respectively); there was minimal gas trapping (residual volume z-score 1.88 ±1.6) and mildly reduced DLCO (% predicted 78±15%). All MBNW indices were higher than normal and also did not change after two months of treatment: median (IQR) z-score: Scond 3.3 (3.1-4.2) L\textsuperscript{-1}, Sacin 2.8 (2.1-3.8) L\textsuperscript{-1} and LCI 4.6 (2.3-7.8); paired t-test: p=0.2, p=0.4 and p=1.0 for Scond, Sacin and LCI respectively).
<table>
<thead>
<tr>
<th></th>
<th>Raw values</th>
<th>Z-score or % predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of subjects, n = 19</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(male = 11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>62.8 ±9.4</td>
<td>-</td>
</tr>
<tr>
<td>Age asthma onset, years</td>
<td>19 (2-50)</td>
<td>-</td>
</tr>
<tr>
<td>(median (IQR))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma duration, years</td>
<td>38.2 ±22</td>
<td>-</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.69 ±0.10</td>
<td>-</td>
</tr>
<tr>
<td>BMI, kg.m(^{-2}) (median (IQR))</td>
<td>27.6 (25.0-30.8)</td>
<td>-</td>
</tr>
<tr>
<td>ACQ-5</td>
<td>1.03 ±0.92</td>
<td>-</td>
</tr>
<tr>
<td>Pre FEV(_1), L</td>
<td>1.88 ± 0.68</td>
<td>-2.3 ±0.7</td>
</tr>
<tr>
<td>Pre FVC, L</td>
<td>3.30 ± 1.06</td>
<td>-0.9 ±0.9</td>
</tr>
<tr>
<td>Pre FEV(_1)/FVC ratio</td>
<td>0.57 ±0.09</td>
<td>-2.6 ±0.9</td>
</tr>
<tr>
<td>Post FEV(_1), L</td>
<td>2.01 ± 0.72</td>
<td>-2.1 ±0.7</td>
</tr>
<tr>
<td>Post FVC, L</td>
<td>3.46 ± 1.06</td>
<td>-0.6 ±1.0</td>
</tr>
<tr>
<td>Post FEV(_1)/FVC ratio</td>
<td>0.58 ±0.09</td>
<td>-2.5 ±0.9</td>
</tr>
<tr>
<td>FRC, L</td>
<td>3.78 ± 1.00</td>
<td>1.04 ±1.1</td>
</tr>
<tr>
<td>TLC, L</td>
<td>6.39 ± 1.54</td>
<td>0.55 ±1.0</td>
</tr>
<tr>
<td>RV, L</td>
<td>2.96 ± 0.84</td>
<td>1.88 ±1.6</td>
</tr>
<tr>
<td>RV/TLC ratio</td>
<td>0.47 ±0.09</td>
<td>1.3 ±1.2</td>
</tr>
<tr>
<td>DLCO, ml/(min*mmHg)</td>
<td>19.9 ± 6.1</td>
<td>78 ±15%</td>
</tr>
<tr>
<td>KCO, ml/(min<em>mmHg</em>L)</td>
<td>4.24 ± 0.6</td>
<td>92 ±16%</td>
</tr>
<tr>
<td>VA, L</td>
<td>4.73 ± 1.43</td>
<td>82 ±18%</td>
</tr>
<tr>
<td>Scond L(^{-1}) (median (IQR))</td>
<td>0.048 (0.042-0.073)</td>
<td>3.55 (2.88-4.125)</td>
</tr>
<tr>
<td>Sacin L(^{-1}) (median (IQR))</td>
<td>0.188 (0.124-0.230)</td>
<td>2.80 (2.08-3.83)</td>
</tr>
<tr>
<td>LCI (median (IQR))</td>
<td>9.6 (8.4-13.9)</td>
<td>3.10 (1.98-7.75)</td>
</tr>
<tr>
<td>B/A ratio (median (IQR))</td>
<td>0.52 (0.44-0.77)</td>
<td>-1.18 (-0.02- -1.65)</td>
</tr>
<tr>
<td>K, cmH(_2)O(^{-1}) (median (IQR))</td>
<td>0.197 (0.131-0.267)</td>
<td>1.58 (-1.08-3.43)</td>
</tr>
</tbody>
</table>
Data presented as mean ± SD unless otherwise stated. BMI: body mass index; ACQ-5: Asthma Control Questionnaire-5; FEV$_1$: forced expiratory volume in one second; FVC: forced vital capacity; FRC: functional residual capacity; TLC: total lung capacity; RV: residual volume; DLCO: diffusing capacity; KCO: carbon monoxide transfer-coefficient; VA: alveolar volume; Scond: ventilation heterogeneity in the convection-dependent airways; Sacin: ventilation heterogeneity in the diffusion-dependent airways; LCI: lung clearance index; B/A: index of lung compliance describing the position of the pressure-volume curve with respect to the pressure axis; K: shape factor of the exponential analysis of the pressure-volume curves.
Lung elastic recoil was lower than normal (B/A% \(z\)-score \(\leq\) -1.64) in 5/18 subjects and compliance was higher than normal (K \(z\)-score \(\geq\) 1.64) in 9/18 subjects (figure 3.5). Elastic recoil pressure at functional residual capacity was low at 1.4 (0.8-3.6) cmH\(_2\)O.

Increasing age was associated with lower lung elastic recoil (B/A%, \(r_s=\) -0.52, \(p=0.02\)) and greater lung compliance (K, \(r_s=0.50, p=0.04\)) but not with MBNW indices. Sacin and LCI were both negatively associated with lower lung elastic recoil, B/A% (\(r_s=-0.53, p=0.03\) and \(r_s=-0.52, p=0.03\), respectively) (table 3.2 and figure 3.6). There were no associations between Scond and B/A% (\(r_s=0.28, p=0.3\)) or between any MBNW indices and lung compliance, K (\(r_s=0.18, p=0.5; r_s=0.002, p=1.0\) and \(r_s=0.33, p=0.2\) for correlations between K and Sacin, Scond and LCI, respectively).

Univariate correlations between MBNW indices and other anthropometric data, spirometry and asthma symptoms were also assessed and results are shown in table 3.2. MBNW indices were not related to asthma duration, gender, height, BMI, FEV\(_1\) or FVC. Only increased LCI was associated with older age of asthma onset (\(r_s=0.56, p=0.01\)) and lower FEV\(_1\)/FVC (\(r_s=-0.47, p=0.04\)). An increase in all VH indices was associated with worse symptom control (ACQ-5) (\(r_s=0.76, p<0.0001\) for Scond; \(r_s=0.54, p=0.02\) for Sacin; \(r_s=0.61, p=0.006\) for LCI).
Figure 3.5
Pressure-volume curve in asthma subjects (coloured lines) and healthy controls (grey lines). Healthy control curves were adapted from Turner et al (39) using data from non-smokers >40 years old.
Table 3.2 Univariate correlations between clinical variables, lung elastic recoil and MBNW after two months of high dose inhaled corticosteroid/long-acting beta-agonist treatment.

<table>
<thead>
<tr>
<th></th>
<th>SCOND, L⁻¹</th>
<th>SACIN, L⁻¹</th>
<th>LCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
<td>rs</td>
</tr>
<tr>
<td>Age, years</td>
<td>0.03</td>
<td>0.9</td>
<td>0.21</td>
</tr>
<tr>
<td>Age of asthma onset, years</td>
<td>0.29</td>
<td>0.2</td>
<td>0.40</td>
</tr>
<tr>
<td>Asthma duration, years</td>
<td>-0.26</td>
<td>0.3</td>
<td>-0.27</td>
</tr>
<tr>
<td>Gender</td>
<td>-0.22</td>
<td>0.4</td>
<td>-0.10</td>
</tr>
<tr>
<td>Height, m</td>
<td>-0.12</td>
<td>0.6</td>
<td>-0.14</td>
</tr>
<tr>
<td>BMI, kg.m⁻²</td>
<td>-0.03</td>
<td>0.9</td>
<td>-0.08</td>
</tr>
<tr>
<td>ACQ-5</td>
<td>0.76</td>
<td>&lt;0.0001</td>
<td>0.54</td>
</tr>
<tr>
<td>FEV₁, L</td>
<td>-0.36</td>
<td>0.1</td>
<td>-0.40</td>
</tr>
<tr>
<td>FVC, L</td>
<td>-0.18</td>
<td>0.5</td>
<td>-0.30</td>
</tr>
<tr>
<td>FEV₁/FVC ratio</td>
<td>-0.18</td>
<td>0.5</td>
<td>-0.15</td>
</tr>
<tr>
<td>B/A ratio</td>
<td>-0.28</td>
<td>0.3</td>
<td>-0.53</td>
</tr>
<tr>
<td>K, cmH₂O⁻¹</td>
<td>0.002</td>
<td>1.0</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Scond: ventilation heterogeneity in the convection-dependent airways; Sacin: ventilation heterogeneity in the diffusion-dependent airways; LCI: lung clearance index; BMI: body mass index; ACQ-5: Asthma Control Questionnaire-5; FEV₁: forced expiratory volume in 1 second; FVC: forced vital capacity; B/A: index of lung compliance describing the position of the pressure-volume curve with respect to the pressure axis; K: shape factor of the exponential analysis of the pressure-volume curves. rs: Spearman correlation coefficient; p: p-value. Statistically significant p-value <0.05.
Figure 3.6
Univariate correlations between MBNW indices (Scond,Sacinand LCI) and lung elastic recoil (B/A%).

Scond: ventilation heterogeneity in the convection-dependent airways; Sacin: ventilation heterogeneity in the diffusion-dependent airways; LCI: lung clearance index; B/A: index of lung compliance describing the position of the pressure-volume curve with respect to the pressure axis. $r_s$: Spearman correlation coefficient; p: p-value.
3.4 Discussion

This study shows that in non-smoking asthmatics over the age of 40 years with FAO, uneven ventilation distribution in diffusion-dependent airways (Sacin) was associated with loss of lung elastic recoil but not with increased lung compliance. Conversely, convection-dependent (Scond) ventilation distribution was unrelated to either lung elastic recoil or compliance. These findings suggest the mechanical properties of the lung tissue are an important determinant of peripheral airway function in older people with asthma and FAO. Furthermore, despite maximal ICS/LABA treatment, abnormal spirometry and ventilation distribution or increased heterogeneity (VH) did not change over a two-month period, suggesting a steroid-unresponsive process. Moreover abnormal or increased VH in the small airways was associated with poor symptom control, implying that small airway function plays a role in the clinical manifestation and expression of symptoms in asthma with FAO.

B/A represents the position of the pressure-volume relationship, but not the shape of the curve (K). B/A and K however, were weakly correlated in our study (r=-0.53, p=0.03). There are no data on the functional, clinical or pathological correlates of B/A and therefore, it is difficult to be certain what the relationship between B/A and Sacin is due to. We speculate that the loss of lung recoil could alter diffusion-dependent ventilation and make it more heterogeneous if, as computational modeling suggests, Sacin is determined by heterogeneity in the cross-sectional areas of the intra-acinar airway openings (10). Elastic recoil arises from the elastic forces generated by alveoli and intra-acinar airways. Colebatch et al suggested that a loss of lung elastic
recoil may be manifested as a left-ward shift of the P-V curve (i.e. reduced B/A\%) (19) or an increase in K or both (33). If reduced lung elastic recoil due to asthma were heterogeneously distributed throughout the lungs, then this could increase acinar VH, if it resulted in alveolar and intra-acinar airway diameters being more variable. Both would increase the variability in specific ventilation, hence VH overall. Conversely, a global loss of elastic recoil could also increase overall VH, either by amplifying normal airway structural asymmetry and/or by bringing some airways towards closure, thus changing the distribution of airway closure.

The loss of lung elastic recoil in asthma is likely due to changes in the structure of the lung tissue (14, 40, 41). Gelb et al reported a heterogeneous distribution of alveolar sizes in the post mortem histologic examination of lungs of asthmatic non-smokers who had died of non-asthma related illness (14, 40). Microscopic examination confirmed mild, diffuse centri-lobular emphysema-like changes, predominantly in the upper to middle lobes, as well as areas of normal lung parenchyma (14, 40). However, the nature of the ultra-structural changes to the lung parenchyma, i.e. potential changes in elastin, collagen and proteoglycan structure, that underlie the emphysema-like changes are unknown. The variability in the histo-pathological abnormalities within an asthmatic lung make it likely that the distribution of lung elastic recoil pressures will also be distributed heterogeneously, thereby increasing VH. Age-related structural changes such as enlargement of the airspaces, alveolar wall thickening and reduced number of peripheral airways (42) may also be distributed unevenly, further contributing to increased VH.
Loss of lung elastic recoil (19), which is known to occur with age, may explain the resultant increase in closing volume and residual volume/total lung capacity (RV/TLC) that occurs with increasing age (19). There was a relationship between age and indices of lung elastic recoil in these subjects and this relationship is discussed in detail in Chapter 5.

K was unrelated to any VH indices in this study. K is an index of alveolar distensibility and is related to alveolar size (38, 43), which is typically increased in the presence of emphysema in smokers (44). K has been reported to be increased in asthmatic non-smokers, but to a lesser extent compared to that seen in emphysema (43). This is consistent with Gelb’s findings discussed above, where there may be microscopic emphysema-like changes, but not macroscopic emphysema (14, 40). Furthermore, B/A%, is not associated with the degree of emphysema in smokers (44). The lack of any relationships between K and VH indices suggest that alveolar distensibility does not affect ventilation distribution in this cohort. The lack of a relationship between K or B/A% and Scond might be due to a greater influence of airway remodelling (rather than lung parenchymal changes) on Scond.

All VH indices (Scond, Sacin and LCI) in our study were increased after adjustment for age, gender and height, given the high mean Z-scores for those indices. Age is the strongest predictor of VH indices in healthy subjects (12, 30, 45). However, none of the VH indices were related to age, either as unadjusted raw values or as Z scores. The narrow age range in our cohort may have influenced this (they are mostly between the ages of 55-75 years).
Therefore, Sacin increases in older asthmatics due to reasons other than simply ageing in the presence of longstanding asthma. We found that LCI was greater, if asthma symptoms first appeared when older. Therefore, the pathological changes that occur when asthma starts may be different with increased age e.g. different inflammatory and remodeling processes, or that the process are the same but the functional consequences differ due to the lung being older. Both age of onset of asthma, and VH indices have important clinical associations. Increased VH indices correlated with worse symptom control (ACQ-5), in keeping with results from previous asthma studies of younger patients with less airflow obstruction (8). Furthermore, adult-onset asthma is associated with decreased lung function and frequent exacerbations (46).

Limitations to our study include the use of raw values rather than predicted values for analyses of MBNW indices. Raw values were used because when data was analysed using two currently available predictive equations (30, 45) (data not shown), the predicted values from these equations were markedly different. One of the predictive equations (30) used the same MBNW device as our study, however the upper age limit of subjects in this study was only 71 years and they also used a multiplying factor of the tidal volume to calculate their MBNW indices. The other predictive equation (45), although studied older subjects >80 years used a different MBNW device (bag-in box).

Statistical analysis was performed using pre-bronchodilator rather than post-bronchodilator measurements. For practical reasons and due to the invasive nature of the test, lung elastic recoil measurements were only performed pre-
bronchodilator. Furthermore, the effect of bronchodilators on lung elastic recoil is controversial. Lung elastic recoil does not change post-bronchodilator therapy in stable asthma (20) however may improve post-bronchodilator during an asthma exacerbation (47, 48) and once asthma control has been optimized and improved (14). The effect of bronchodilators on VH in asthma with FAO is not known, however in COPD there appears to be no effect (49). The effect of medications prior to study enrolment were not accounted for however subjects were treated with a standardized ICS/LABA dose for a two-month period prior to lung elastic recoil measurements. The study treatment also appeared to have no effect on lung function and symptoms, as there were no significant differences in spirometry or MBNW indices and ACQ-5 scores over the two-month study period.

In summary, we found that a loss of elastic recoil, but not lung compliance, was associated with increased VH in diffusion-dependent airways in older asthmatics with FAO. The mechanisms causing loss of lung elastic recoil in asthma needs further investigation as it may provide insight into causes of small airway dysfunction in asthmatics who develop FAO despite negligible smoking history. The role of inflammation needs further exploration, as high dose ICS/LABA did not change lung function over a two-month period, suggesting steroid unresponsiveness. Whether immunosenescence, asthma or both contribute to the underlying cellular mechanisms remains elusive. This may represent a potential pathway by which the asthma-COPD overlap phenotype is manifest and therefore highlights the need for development of novel treatments that target loss of elastic recoil in asthma.
References


Chapter 4

4 The Contributions of Small Airway Obstruction and Reduced Lung Elastic Recoil to Airflow Obstruction in Asthma
4.1 Introduction

Asthma is characterised by variable airflow obstruction that occurs intermittently and in response to inhaled stimuli. However, airflow obstruction may persist despite bronchodilator inhalation or optimal therapy with inhaled corticosteroids and long-acting bronchodilators, i.e. it may become fixed and irreversible (1). Fixed airflow obstruction (FAO) is associated with longstanding disease duration (2), persistent airway hyper-responsiveness AHR (3), persistent eosinophilic (3) or neutrophilic airway inflammation (4) and mucous hyper-secretion (5). The underlying mechanisms of FAO however, are poorly understood.

FAO in asthma is most commonly attributed to airway remodelling. Airway remodelling refers to alteration of the structural organisation of the airway wall, which includes the entire wall from the mucosa through to the adventitia. This results in an increase in airway wall thickness and altered airway mechanical properties. Hence, airway remodelling is the most well known explanation for impaired spirometry in asthma. However, there is strong evidence that in addition to airway remodelling, mechanical and structural changes to the lung parenchyma (6, 7), a type of ‘lung remodelling’, contributes to FAO in asthma, particularly in older subjects.

The elastic recoil of the lung provides outward recoil to the airways and alveolar driving pressure during exhalation. Therefore, any processes that reduce lung elastic recoil pressure will also reduce airway calibre at any given lung volume i.e. increase airway resistance (8, 9) and increase airway closure. Consequently reduced lung elastic recoil will have the same effects on
spirometry as airway remodelling. Reduced lung elastic recoil in asthmatics has been described repeatedly (8-12) however the underlying cause is unknown.

Analysis of lung pressure-volume (P-V) curves by mathematical curve fitting (13) and construction of maximal flow static recoil (MFSR) curves (14) produces indices of the mechanical properties of the lung and airways, respectively. Indices derived from the MFSR curves represent changes in peripheral airway calibre (conductance) and increased collapsibility of the airways during forced expiratory flow (14). Determining whether the lungs elastic or intrinsic airway properties contribute to expiratory flow limitation can be assessed using these methods.

Therefore, the aim of this study was to determine the contributions of lung elastic recoil and peripheral airway mechanics, to the impairment of lung function measured by spirometry (FEV₁/FVC ratio) and by forced oscillatory impedance, in non-smoking older adults with asthma. We hypothesised that reduced lung elastic recoil and abnormal airway mechanics contribute independently to airflow obstruction.

4.2 Methods
4.2.1 Subjects
Adult subjects were recruited from a volunteer database and from referrals by local respiratory physicians. Subjects were eligible if they were aged 40 years or older, as abnormalities in elastic recoil are increasingly common from middle age onwards (12). All subjects had a physician-confirmed diagnosis of asthma based on typical symptoms, previous evidence of variable airflow
obstruction and a history of asthma medication use. Exclusion criteria were a past smoking history of ≥5 pack-years, an asthma exacerbation within the past 8 weeks, the presence of cardiac disease or respiratory disease other than asthma. Written informed consent was obtained from all subjects. Ethics approval was granted by the Sydney Local Health District Human Research Ethics Committee (#HRECT/14/CRGH/75).

4.2.2 Study design
Eligible subjects were treated with maximal dose inhaled corticosteroid/long-acting β-agonist (ICS/LABA) using Fluticasone/Eformoterol 250μg/10μg metered dose inhaler via a holding chamber, two puffs twice daily for two months. The rationale was to ensure that any abnormalities that were related to steroid-responsive inflammation would be eliminated by this standardised treatment (15). Subjects answered a standard clinical and symptom questionnaire, Asthma Control Questionnaire (ACQ-5) (16). After one month of ICS/LABA treatment, pre and post-bronchodilator spirometry was performed. After two months, in a single session subjects underwent measurements of standard lung function, skin prick tests, bronchial challenge, forced oscillation technique and lung elastic recoil. If subjects developed an exacerbation during the study period, lung function testing was delayed for a minimum period of one month after clinical improvement. ICS/LABA medication was withheld for at least 24 h and short acting β2-agonist medication for at least 6 h prior to testing.
4.2.3 Lung function tests

4.2.3.1 Spirometry, lung volumes and diffusing capacity.
Spirometry, lung volumes and diffusing capacity were performed using a Medisoft BodyBox 5500 (Medisoft Corp; Sorrines, Belgium) according to ATS/ERS guidelines (17-19). Predicted values were reported according to published reference equations (20-22).

4.2.3.2 Skin Prick Test
A skin prick test (23) to assess atopy was performed to a standard set of 8 allergens. Subjects were instructed to withhold antihistamines for 48h prior to the skin prick test. A positive reaction was a wheal size of ≥ 4mm and atopy was defined as ≤1 positive reaction.

4.2.3.3 Bronchial challenge testing
Methacholine challenge tests were performed using handheld DeVilbiss No 45 nebuliser (Sunrise Medical; Carlsbad, California), with doses ranging from 0.1 to 12.2 μmol using the Rapid Method (24). Response to challenge was measured by the dose-response slope (DRS), which is calculated as ( [% fall in FEV1/μmol methacholine] +3). AHR was defined as DRS >8% fall in FEV1/μmol (equivalent to FEV1 provocative dose causing a 20% reduction in FEV1 <4μmol).

4.2.3.4 Forced oscillation technique (FOT)
Respiratory system impedance was measured by the forced oscillation technique (tremoFlow C-100 device, Thorasys Medical Systems, Montreal, QC, Canada), with a multifrequency forcing signal composed of 5, 11 and 19Hz. Recordings were made with patients seated, wearing a nose clip and supporting the cheeks and under the chin with their hands. After stable tidal
breathing, data was acquired for 60 seconds. Technically acceptable recordings were obtained in triplicate. Respiratory system resistance for the 5Hz signal ($R_s$) and respiratory system reactance at 5Hz ($X_s$) were used for analyses. The predicted values were those of Oostveen et al (25).

4.2.3.5 Lung pressure-volume curves
Pressure-volume curves were constructed as described in Chapter 3.

4.2.3.6 Maximal flow static recoil (MFSR) curves
MFSR curves were constructed as previously described using maximal expiratory airflow obtained from the flow-volume curves against static lung elastic recoil pressure from the P-V curve at corresponding lung volumes (12). The slope of the MFSR curve between 70% and 30% of the forced vital capacity (FVC) and X-axis intercept were determined by least-squares linear regression. According to the analysis of Leaver et al (14) the slope of the line represents conductance (calibre) of the upstream segment ($G_{us}$) i.e. between the alveolus and flow limiting segment, while the X-axis intercept represents collapsibility of the flow limiting segment ($C_{FLS}$). The X-axis intercepts are usually negative in healthy subjects and may increase and become positive in airways disease, i.e. airway collapse may occur at higher pressure.

4.2.4 Statistical analyses
Data are presented as means ±SD for normally distributed data and as medians (interquartile range IQR) for non-normally distributed data. Spirometry, FOT and lung elastic recoil indices were expressed as z-scores and percent predicted. Univariate relationships between dependent variables (FEV$_1$/FVC ratio and $R_s$) and lung elastic recoil and MFSR indices were
examined using Spearman correlation coefficients. Pre-bronchodilator spirometry values were used for analysis as this was performed on the same day as lung elastic recoil measurements. Stepwise linear regression was used to determine the independent predictors of elastic recoil and MFSR abnormalities to FEV₁/FVC or R₅. A threshold p-value of <0.2 was used to identify potential predictors for the multiple-linear regression model. The distributions of the residuals of the linear regression model were checked for normality. P<0.05 was considered significant.

4.3 Results

4.3.1 Subjects and lung function data
A total of 21 subjects were enrolled and two were excluded because they were unable to complete the lung function tests. Elastic recoil measurements were performed in 18 of the remaining 19 subjects. Anthropometric and lung function data are presented in table 4.1. Subjects were older, with a mean age of 63 ±9 years and long duration of asthma (38.2 ±22years). Most subjects were taking regular asthma medications prior to enrolment – 18 were taking ICS (in LABA combination or as a single inhaler) and five were taking LAMA (one subject in LABA/LAMA combination). One subject was on long-term low dose oral corticosteroids (Prednisone dose 5 mg) for treatment of rheumatoid arthritis. Five subjects were ex-smokers with 2.2 ±2.5 pack-years smoking history. Subjects had mildly uncontrolled asthma symptoms (ACQ-5 1.03 ±0.92). Atopy was present in 13/19 subjects and 8/18 subjects demonstrated AHR (FEV₁ was <50% of predicted in 1 subject which precluded bronchial challenge testing). There was mild-moderate impairment of spirometry and
respiratory impedance. Spirometry did not change after the two-month study period however respiratory resistance increased (see chapter 3 results).
Table 4.1 Anthropometric and lung function data.

<table>
<thead>
<tr>
<th>Number of subjects, n = 19 (male = 11)</th>
<th>Raw values</th>
<th>Z-score or % predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>62.8 ±9.4</td>
<td>-</td>
</tr>
<tr>
<td>Age asthma onset, years (median (IQR))</td>
<td>19 (2-50)</td>
<td>-</td>
</tr>
<tr>
<td>Asthma duration, years</td>
<td>38.2 ±22</td>
<td>-</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.69 ±0.10</td>
<td>-</td>
</tr>
<tr>
<td>BMI, kg.m$^{-2}$ (median (IQR))</td>
<td>27.6 (25.0-30.8)</td>
<td>-</td>
</tr>
<tr>
<td>Pre FEV$_1$, L</td>
<td>1.88 ± 0.68</td>
<td>-2.3 ±0.7</td>
</tr>
<tr>
<td>Pre FVC, L</td>
<td>3.27 ± 1.09</td>
<td>-1.0 ±1.1</td>
</tr>
<tr>
<td>Pre FEV$_1$/FVC ratio</td>
<td>0.58 ±0.10</td>
<td>-2.4 ±0.9</td>
</tr>
<tr>
<td>Post FEV$_1$, L</td>
<td>2.01 ± 0.72</td>
<td>-2.1 ±0.7</td>
</tr>
<tr>
<td>Post FVC, L</td>
<td>3.46 ± 1.06</td>
<td>-0.6 ±1.0</td>
</tr>
<tr>
<td>Post FEV$_1$/FVC ratio</td>
<td>0.58 ±0.09</td>
<td>-2.5 ±0.9</td>
</tr>
<tr>
<td>FRC, L</td>
<td>3.78 ± 1.00</td>
<td>1.04 ±1.1</td>
</tr>
<tr>
<td>TLC, L</td>
<td>6.39 ± 1.54</td>
<td>0.55 ±1.0</td>
</tr>
<tr>
<td>RV, L</td>
<td>2.96 ± 0.84</td>
<td>1.88 ±1.6</td>
</tr>
<tr>
<td>RV/TLC ratio</td>
<td>0.47 ±0.09</td>
<td>1.3 ±1.2</td>
</tr>
<tr>
<td>DLCO, ml/min/mmHg</td>
<td>19.9 ± 6.1</td>
<td>78 ±15</td>
</tr>
<tr>
<td>DLCO/VA</td>
<td>4.24 ± 0.6</td>
<td>92 ±16%</td>
</tr>
<tr>
<td>VA, L</td>
<td>4.73 ± 1.43</td>
<td>82 ±18%</td>
</tr>
<tr>
<td>$R_s$, hPa.s.L$^{-1}$ (median (IQR))</td>
<td>5.8 (4.3-7.5)</td>
<td>2.7 (1.8-3.2)</td>
</tr>
<tr>
<td>$X_s$, hPa.s.L$^{-1}$ (median (IQR))</td>
<td>-2.7 (-1.8 - -5.1)</td>
<td>-3.9(-2.0--7.3)</td>
</tr>
<tr>
<td>A, L</td>
<td>6.55 ±1.59</td>
<td>-</td>
</tr>
<tr>
<td>B, L</td>
<td>3.93 ±1.94</td>
<td>-</td>
</tr>
<tr>
<td>K, cmH$_2$O$^{-1}$ (median (IQR))</td>
<td>0.197(0.131-0.267)</td>
<td>1.58(-1.08-3.43)</td>
</tr>
<tr>
<td>B/A ratio (median (IQR))</td>
<td>0.52 (0.44-0.77)</td>
<td>-1.18(-0.02--1.65)</td>
</tr>
<tr>
<td>$G_{US}$, L.s.cmH$_2$O$^{-1}$ (median (IQR))</td>
<td>0.34 (0.27-0.480)</td>
<td>-</td>
</tr>
<tr>
<td>$C_{FLS}$, cmH$_2$O (median (IQR))</td>
<td>0.69 (-0.09-3.16)</td>
<td>-</td>
</tr>
</tbody>
</table>
$R_s$: respiratory system resistance at 5Hz; $X_s$: respiratory system reactance at 5Hz; $A$: asymptote of the exponential analysis of pressure-volume curves; $B$: distance on the Y-axis between $A$ and the extrapolated Y-axis intercept of the pressure-curve; $K$: shape factor of the exponential analysis of the pressure volume-volume curves, $B/A$: index of lung compliance describing the position of the pressure-volume curve with respect to the pressure axis, $G_{US}$: conductance of the upstream segment determined from MFSR curves, $C_{FLS}$: collapsibility of the flow limiting segment determined from MFSR curves. Data presented as mean ±SD unless otherwise stated.
The PV curves are shown in chapter 3. In five subjects, the lower limit of the PV curve was fitted to a volume ≥60% of TLC (rather than 50%) as they were mild-moderately hyperinflated with a FRC > 50% of TLC (mean ±SD % predicted FRC 134±11%, for these five subjects). Mean FRC for these five subjects was also greater than mean FRC for the whole group (117±19%). Median (IQR) values for B/A and K were 0.52 (0.44-0.77) and 0.197 (0.131-0.267) cmH₂O⁻¹, respectively. Lung elastic recoil was lower than normal (B/A% z-score ≤-1.64) in 5/18 subjects and compliance was higher than normal (K z-score ≥1.64) in 9/18 subjects. There was a moderate correlation between B/A and K z-scores ($r_s$=-0.54, p=0.02). The MFSR curve for each subject is shown in figure 4.1.
Figure 4.1

Maximal flow static recoil (MFSR) curves in asthma subjects.
4.3.2 Univariate correlations with spirometric ratio

Spirometric ratio (FEV₁/FVC) z-score correlated negatively with K z-score ($r_s=-0.60$, $p=0.008$) (figure 4.2). FEV₁/FVC z-score also correlated negatively with age ($r_s=-0.49$, $p=0.03$) and positively with BMI ($r_s=0.65$, $p=0.003$) (table 4.2) but was unrelated to asthma duration, B/A z-score or MSFR indices ($C_{FLS}$ and $G_{US}$).
Table 4.2 Correlation between clinical and lung elastic recoil parameters with measures of airflow obstruction.

<table>
<thead>
<tr>
<th>Variable</th>
<th>FEV₁/FVC (z-score)</th>
<th>R₅ (z-score)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rₛ</td>
<td>P</td>
</tr>
<tr>
<td>Age, years</td>
<td>-0.49</td>
<td>0.03</td>
</tr>
<tr>
<td>Asthma duration, years</td>
<td>-0.15</td>
<td>0.54</td>
</tr>
<tr>
<td>BMI, kg.m⁻²</td>
<td>0.65</td>
<td>0.003</td>
</tr>
<tr>
<td>B/A ratio (z-score)</td>
<td>0.21</td>
<td>0.40</td>
</tr>
<tr>
<td>K (z-score)</td>
<td>-0.60</td>
<td>0.008</td>
</tr>
<tr>
<td>Gₚₜ, L.s.cmH₂O⁻¹</td>
<td>-0.006</td>
<td>0.98</td>
</tr>
<tr>
<td>Cₚₜ, cmH₂O⁻¹</td>
<td>0.33</td>
<td>0.18</td>
</tr>
</tbody>
</table>

BMI: Body Mass Index; FEV₁/FVC: Forced Expiratory Volume in 1 second/Forced Vital Capacity; R₅: respiratory system resistance at 5Hz; B/A: index of lung compliance describing the position of the pressure-volume curve with respect to the pressure axis; K: shape factor of the exponential analysis of the pressure-volume curves, Gₚₜ: conductance of the upstream segment determined from MFSR curves, Cₚₜ: collapsibility of the flow limiting segment determined from MFSR curves. rₛ: Spearman correlation coefficient; p: p-value. Statistically significant p-value <0.05.
Figure 4.2

Univariate correlation between airflow obstruction (forced expiratory volume in 1 second/forced vital capacity, FEV₁/FVC) and lung compliance, K.
4.3.3 Univariate correlations with forced oscillatory impedance

There was a negative correlation between $R_5$ and B/A z-scores ($r_s=-0.52$, $p=0.026$) (figure 4.3) but no correlation with K z-score. $R_5$ z-score did not correlate with age, BMI, asthma duration or MFSR indices. There was a strong negative correlation between $R_5$ and $X_5$ z-score ($r_s=-0.62$, $p=0.005$). $X_5$ z-score did not correlate with B/A or K z-scores, age, BMI, asthma duration and $C_{FLS}$. There was a positive correlation with $G_{ua}$ ($r_s=0.50$, $p=0.04$).

![Figure 4.3](image)

Univariate correlation between airway narrowing (resistance, $R_5$) and lung elastic recoil, B/A.
4.3.4 Multivariate correlations with spirometry and forced oscillation impedance

Table 4.3 show the results from multiple linear regression analyses. The following co-variates were used in the model: age, BMI, disease duration, K z-score, B/A z-score, GUS and CFLS. The only independent predictor of FEV₁/FVC z-score was K z-score (model $r^2=0.38$, p=0.007). B/A z-score and GUS were independent predictors of $R_5$ z-score (model $r^2=0.44$, p=0.01). The only independent predictor of $X_5$ z-score was GUS (model $r^2=0.38$, p=0.006).

Table 4.3 Stepwise multiple linear regression to identify the independent predictors of forced expiratory volume in 1 second/forced vital capacity (FEV₁/FVC) and resistance at 5Hz ($R_5$).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>$\beta$-coefficient ± SE</th>
<th>Partial $r^2$</th>
<th>Adjusted model $r^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁/FVC</td>
<td>K (z-score)</td>
<td>-0.62 ±0.06</td>
<td>0.38</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_5$</td>
<td>B/A (z-score)</td>
<td>-0.63 ±0.18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GUS</td>
<td>-0.45 ±2.26</td>
<td>0.44</td>
<td>0.37</td>
</tr>
</tbody>
</table>
4.4 Discussion
In this study, older non-smokers with asthma demonstrate FAO, increased resistance and loss of lung elastic recoil. Importantly airflow obstruction is associated with loss of lung elastic recoil, independent of intrinsic airway narrowing. This suggests there are changes occurring outside the airway wall in the surrounding lung tissue, likely contributing to the loss of lung elastic recoil. Furthermore, the ageing process is an additional factor contributing to FAO. To our knowledge the novel correlations between these measures of airflow obstruction (FEV₁/FVC and R₅) and lung elastic recoil (K and B/A) have not been reported in the literature, in particular in older non-smokers with asthma and FAO.

Our results are consistent with findings from previous studies showing a loss of lung elastic recoil in asthma (8, 9, 12, 26, 27), especially in older people (12). Gelb et al recruited older clinically stable asthmatics with moderate airflow obstruction and showed an unexpected significant loss of lung elastic recoil (12). The P-V curves were compared to age matched controls and were left shifted, which suggested that as a group, their elastic recoil was abnormal. Our P-V curves were also leftward shifted which we quantified using the index B/A (13, 28). We also quantified loss of lung elastic recoil using the index K (13, 28). The high z-scores indicate a loss of elastic recoil compared to healthy (28) subjects and compared to that reported previously in asthmatic adults (8). Our asthma subjects were older and had worse lung function compared to this asthma study (8), which likely explains the differences in findings. In addition, K values in our study are similar to that measured in post
mortem lungs in smokers with emphysema (29, 30), in which elastic recoil is reduced as a result of alveolar destruction.

The cause of loss of elastic recoil in asthma is not clear however there is limited evidence from post-mortem (6, 7) and imaging studies (31-35) confirming alteration in lung tissue. Microscopic examination of post mortem lungs from three lifelong non-smokers, who died of causes unrelated to asthma showed diffuse, mild centrilobular emphysema with upper-lobe predominance (6). Abnormal alveolar attachments and decreased elastic fibre content has also been shown in post mortem lung tissue in fatal asthma (7). Furthermore, decreased lung parenchymal density (lung attenuation) from computer tomography scans has been described qualitatively and measured quantitatively in asthma (31-35). We can speculate that decreased lung attenuation is consistent with alveolar space enlargement and consequent reduced lung elastic recoil (36). K is thought to resemble alveolar size (30, 37), with increased K reflecting an increase in alveolar size or alveolar enlargement. Increased K in our study supports the concept of alveolar enlargement however the underlying pathological process is unknown. We cannot confirm emphysema in our subjects however mean % predicted DLCO was reduced (mean ±SD: 78 ±15%). Additionally % predicted DLCO was lower in those who had a K z-score greater than 1.64 SD from the predicted value, compared to those with a K z-score less than 1.64 SD (73 ±18% and 81 ±11%, respectively). Reduced DLCO is not typically associated with asthma however we could speculate that this result reflects alteration in the lung tissue resulting in subsequent loss of elastic recoil.
The ageing process is an additional factor contributing to FAO in asthma, highlighted by the negative correlation between FEV₁/FVC and age, which still exists even after accounting for age. It is known that lung elastic recoil declines with age (38-41) and has been attributed to changes in the alveolar wall, collagen and elastic fibres (42-46). Uniform reduction in the number and thickness of elastic fibres has been shown in the ageing human lung. These changes were confined to the alveolar ducts and alveoli, resulting in dilatation of the alveolar ducts (46). Subsequently lung over-inflation may occur and can explain the emphysema-like physiological abnormalities seen in older people such as increased FRC and RV (40). New insights suggest that chronic obstructive disease starts in childhood or in utero with genetic, environmental and lifestyle factors influencing lung function early in life (47-50). Extrapolating from these studies, we can speculate that lung elastic recoil may also be reduced early in life therefore continue to decline from a lower starting point.

B/A and K show a moderate correlation with age ($r_s=0.50$, $p=0.035$ and $r_s=-0.54$, $p=0.02$ for B/A and K, respectively) and when age is taken into account using z-scores, there is a similar trend ($r_s=0.44$, $p=0.07$ and $r_s=-0.45$, $p=0.06$ for B/A and K z-scores, respectively). However there is no correlation between the age of asthma onset or disease duration with FEV₁/FVC, B/A or K. This lack of relationship suggests the ageing process rather than disease contributes to these functional abnormalities. Nevertheless, it is likely the combination of ageing and disease contributing to loss of lung elastic recoil and FAO in older people with asthma. Inflammation may play a role as
chronic airway inflammation is transmural therefore affecting the full thickness of the airways and surrounding lung tissue (51).

The loss of lung elastic recoil contributes to airflow obstruction independent of intrinsic airway narrowing. This is consistent with findings by Gelb et al, who found maximal expiratory airflow limitation at low lung volumes to be due to loss of lung elastic recoil alone in four elderly asthmatics (12). We found different indices of airflow obstruction correlated with different indices of loss of elastic recoil, namely FEV₁/FVC and K and R₅ and B/A, independent of age, asthma duration, BMI and C_FLS. Surprisingly we also found that loss of elastic recoil was associated with lower C_FLS (i.e.: less collapsible airways); B/A: rₛ=0.77, p<0.0001 and K: rₛ=-0.58, p=0.01. This suggests that in those with higher C_FLS (i.e.: more collapsible airways) perhaps loss of elastic recoil is unlikely to be the main contributor to airway obstruction, instead airway obstruction is more likely to be due to intrinsic airway narrowing (i.e. altered mechanics of the airway itself). However, the contribution of loss of elastic recoil to FAO in asthma cannot be ignored. The lung is a complex structure and these different correlations highlight the heterogeneous nature of asthma.

Limitations in our study include the effect of prior medications, construction of the PV curves and lack of healthy controls and imaging or lung tissue data. Any effects of prior medications have not been accounted for, however treatment over the two-month study period was standardized to minimize this cofounder. Construction of the PV curves in five subjects only included points to a volume ≥ 60% of TLC, rather than 50% of TLC, due to these subjects being hyperinflated. Two of the five subjects had B/A z-scores less than 1.64
SD and four subjects had K z-scores greater than 1.64 SD. This may introduce bias as the PV curve would be more left shifted and show greater curvature (i.e.: B/A reduced and K increased) therefore the remaining 15 PV curves were not adjusted to the same volume. A healthy control group was not included in our study as this was a mechanistic study and we wanted to determine physiological mechanisms in the diseased state. Our lung function results are however similar to previous studies in asthmatics of a similar age. Chest imaging and lung tissue would also be helpful to confirm alterations in lung tissue however this data is not available for our cohort.

In conclusion we have further evidence that FAO in older non-smokers with asthma can be attributed to loss of lung elastic recoil and not just airway remodelling. Our findings also suggest there are similarities in the underlying mechanisms of FAO in non-smokers with asthma and emphysema, as the loss of lung elastic recoil probably reflects physiological alveolar-lung parenchymal abnormalities. This study confirms the heterogeneous nature of asthma presentation and clinical expression inferring there are different pathological mechanisms driving the disease. Research into the underlying cellular mechanisms that relate loss of lung elastic recoil to airway narrowing needs further investigation. Perhaps an alternate paradigm for asthma needs to be considered and the contribution of ‘lung remodelling’ warrants further study with potential implications on prevention of FAO in asthma.
References


Chapter 5

Steroid insensitive fixed airflow obstruction is not related to airway inflammation in older non-smokers with asthma.
5.1 Introduction
Irreversible or fixed airflow obstruction (FAO) can develop in long-standing asthma despite no or minimal smoking history and is associated with moderate to severe disease (1, 2). The mechanisms of FAO in asthma are poorly understood; therefore prevention and treatment remain a challenge. Inhaled and/or oral corticosteroids improve lung function and reduce exacerbations, yet may not necessarily prevent FAO from occurring (3, 4). Asthma severity (1, 5) and FAO development may be attributed to corticosteroid resistance or insensitivity resulting in persistent airway inflammation (6) and structural airway changes. Both eosinophilic and neutrophilic inflammation may be associated with lung function impairment and FAO in asthma, however evidence is limited and contradictory (4, 7).

In this prospective study, we investigated whether lung function impairment in older non-smokers with long-standing asthma and FAO is associated with the airway inflammation which remains after treatment with maximal dose inhaled corticosteroid (ICS). We hypothesized that the degree of lung function abnormalities would positively correlate with persistent airway inflammation in patients with asthma and FAO, thereby providing a potential mechanism for the development of FAO and its apparent steroid insensitivity.

5.2 Methods
Patients were >40 years old, non-smokers or had a negligible smoking history with a respiratory physician diagnosis of asthma. All patients were treated with a standardized maximal dose of ICS/long-acting beta-agonist (ICS/LABA) using fluticasone/eformoterol 250μg/10μg metered dose inhaler via a holding
chamber, two puffs twice daily, if not already taking this treatment. A baseline test skin prick test to common allergens was performed to assess atopic status (8). During enrolment and after two months of treatment, patients completed a symptom questionnaire (Asthma Control Questionnaire, ACQ-5) (9) and performed pre-bronchodilator lung function measurements. Measurements included spirometry (10) and the forced oscillation technique (FOT) to derive airway resistance ($R_5$) and reactance ($X_5$) at 5 Hz (11), as described in previous chapters. After two months of treatment an oesophageal balloon was used to derive the pressure-volume (P-V) curve to assess the elastic recoil properties of the lung via the indices K, reflecting lung compliance, and B/A, reflecting lung elastic recoil (12), as described in chapter three. FAO was assessed following one month of treatment and was defined as a spirometric ratio less than the lower limit of normal (13) and/or a <200 ml and <12% change in spirometry post-bronchodilator (400mcg inhaled salbutamol) (14). ICS/LABA medication was withheld for at least 24 h and short acting beta-agonist medication for at least 6 h prior to testing.

Following two months of ICS/LABA treatment and within a week of the lung function measurements, patients then underwent bronchoscopy with bronchoalveolar lavage (BAL) from the right middle lobe (15-21). Bronchoscopy was performed as per standard clinical practice. Patients were pre-medicated with 2.5-5 mg Salbutamol via nebulizer and local anaesthetic gel and spray was applied to the nose and throat. Intravenous midazolam (1-5 mg) and alfentanil (100-500 mcg) was used to sedate the patients and doses were titrated as necessary. Bronchoscope intubation was via the oral or nasal
route and 1% xylocaine was applied to the vocal cords, carina and right main bronchus. The bronchoscope was wedged into the right middle lobe sub-segmental bronchus and BAL was performed using x4 60 ml aliquots of 0.9% saline pre-warmed to 37°C (22). Each aliquot was immediately recovered using gentle continuous negative pressure (23). BAL samples were immediately placed on ice then combined for processing and strained through a monolayer of surgical gauze to remove mucus (15). BAL was centrifuged at 1,500 rpm for 5 minutes at 4 °C and cell-free supernatant was aliquoted and stored at -80 °C until assayed (19). Cell counts were performed using a haemocytometer and trypan blue exclusion testing (16). Differential cell counts were obtained on cytopsin preparations, using a Diff-Quik™ stain, counting 400 cells. Concentrations of IL-1b, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, scD40L and TNF-α (Bio-Rad® Bio-Plex Multiplex Immunoassay) were simultaneously evaluated using a commercially available multiplex bead-based sandwich immunoassay kit on the Lumiex Magpix system (Advanced Tissue Regeneration Group at UTS). Assays and data analysis heat maps were generated using DanteR software as previously described (24). Univariate correlations (Spearman rank test) between lung function indices (using z-scores) and BAL samples (using raw values) after two months of treatment were assessed.

5.3 Results
Nineteen patients were recruited (11 male; mean ±SD age 63±9 years, asthma duration 38 ±22 years, height 1.69±0.10 metres, body mass index 28.4±5.8 kilogram/metre²); 18 completed the study. Five patients were ex-
smokers with 2.2 ±2.5 pack-years smoking history and 14/19 patients were atopic. Patients were symptomatic (ACQ-5 1.03 ±0.92) despite taking regular asthma medications prior to enrolment (ICS 18/19; with LABA 18/19; ICS/LABA/long-acting muscarinic antagonist 5/19). One patient was on long-term low dose oral corticosteroids (Prednisone dose 5 mg) for treatment of rheumatoid arthritis.

Post-bronchodilator spirometry after one month of treatment showed moderate FAO (mean ±SD z-score: FEV₁ -2.05 ±0.75, FVC -0.61 ±0.95, FEV₁/FVC -2.46 ±0.90). After two months of treatment FOT indices were abnormal: R₅ (median (IQR) z-score: 2.7(1.8-3.2)) and X₅ (z-score: -3.9(-2.0--7.3)). Spirometry did not change between enrolment and after two months of treatment, however R₅ worsened. Eighteen patients performed lung elastic recoil measurements (median (IQR) z-score: K 1.57(-1.08-3.43) and B/A% -1.18(-1.65--0.02)). Increased compliance was demonstrated in 9/18 patients (K z-score ≥1.64) and loss of elastic recoil in 5/18 (B/A% z-score ≤-1.64).

Eighteen patients performed bronchoscopy and BAL neutrophil and eosinophil cell counts were obtained in 10 patients (mean ±SD: neutrophils 9.1±18.1% and eosinophils 1.9±1.6%). No patients had evidence of neutrophilic airway inflammation whilst 4/10 patients had eosinophilic airway inflammation. BAL cytokines were obtained in 17 patients and results are shown in figure 5.1. BMI, spirometry, FOT and elastic recoil indices did not correlate with BAL neutrophil or eosinophil count and inflammatory cytokines (figure 5.2). Occasionally, statistically significant correlations were observed however these were the result of a single outlier.
Figure 5.1

Cytokine levels measured in bronchoalveolar lavage fluid from each patient. Each row represents a patient and each column represents different cytokines. Red indicates highest levels and bright green lowest levels. IL: interleukin, IFN-γ=interferon gamma, sCD40L: soluble CD40 ligand, TNF-α: tissue necrosis factor alpha.
Univariate relationships between lung function measurements and BAL IL-17a. No significant correlations were demonstrated and similar findings were seen with other cytokines. FEV₁/FVC: forced expiratory volume/forced vital capacity, $X_5$: reactance at 5Hz, $K$: reflects lung compliance, BAL: bronchoalveolar lavage, IL: interleukin. R: Spearman correlation coefficient; P: p-value.
5.4 Discussion
Variable levels of neutrophils, eosinophils and cytokines were detected in this small cohort however there was a disconnect between measures of airway inflammation and lung function. The lack of a relationship in this cohort suggests persisting airway inflammation does not affect lung function following ICS treatment. However, the effect of previous inflammation and inflammation over time on lung function and FAO development remains unknown. A standardized period and dose of ICS treatment was used to minimize potential confounders however most patients were not steroid naïve thus the study treatment may have had minimal additional effect on the inflammatory profile. This is supported by the absence of any significant change in spirometry from enrolment and after the two-month study period. Adherence to study treatment was assessed after one month and at the two-month mark to ensure non-adherence did not play a role.

Somewhat surprisingly and in contrast to previous studies (25), this older cohort did not demonstrate neutrophilic airway inflammation although small subject numbers are a limiting factor. Furthermore, neutrophil activation was not measured and may have been increased due to inhibition of neutrophil apoptosis by inhaled corticosteroids. Despite treatment with high-dose ICS/LABA, eosinophilic inflammation persisted in a few patients and cytokines were still detectable, suggesting a steroid unresponsive inflammatory pathway. The fact that FAO develops despite treatment suggests inhaled corticosteroids may have minimal effect on airway remodelling in older people with asthma. Instead FAO in this cohort may predominantly be due to other
mechanisms such as the loss of elastic recoil observed in this study, which in turn may occur as a result of lung tissue changes (i.e. lung remodelling) (2). Lung tissue changes could be due to proteolytic enzymes disrupting lung parenchyma-terminal bronchiole attachments (26). Inflammation in the lung tissue cannot be ignored, however our study lacks the ability to assess this. Less invasive tests such as a computer tomography (CT) scan to assess for possible lung tissue changes like emphysema was also not done. A recent study demonstrated micro-emphysema, only on microscopic examination of post-mortem asthmatic lungs, which was not evident on CT imaging (2), therefore inclusion of CT imaging may not have be adequate to demonstrate lung tissue changes in our study.

5.5 Conclusion
In summary, this exploratory cross-sectional study has shown a lack of relationship between persistent airway inflammation and lung function impairment following a short period of maximal ICS treatment. However, other cellular mechanisms, lung tissue inflammation and the potential longitudinal effect of inflammation over time in the development of FAO in asthma warrant further investigation.
References


Chapter 6

6 Conclusion
Fixed airflow obstruction (FAO) occurs in non-smokers with asthma (1-4) and the underlying mechanisms are not clear. The small airways are the major site of airflow obstruction (5, 6) and the paradigm underpinning asthma pathogenesis is airway remodelling driven by and/or as a consequence of complex inflammatory pathways (7). However other factors such as the lung tissue (8, 9) also contribute. The overall aim of this thesis was to explore physiological and inflammatory mechanisms contributing to fixed airflow obstruction in older non-smoking adults with asthma. Airway physiology and lung mechanics were assessed using standard lung function tests; the multiple breath nitrogen washout (MBNW) test to determine ventilation heterogeneity; the forced oscillation technique to determine respiratory resistance and reactance; and the oesophageal balloon technique to determine the lungs elastic properties. Inflammation was measured using bronchoalveolar lavage samples. A summary of the main findings from this thesis and future directions are discussed below.

6.1 Functional residual capacity and ventilation heterogeneity measurements between commercial MBNW devices are not comparable.

Functional residual capacity (FRC) is a quality control metric for the MBNW test therefore accuracy of its measurement is paramount. This thesis has shown there are differences in both in vitro and in vivo FRC, as measured by currently available commercial MBNW devices. The pattern of FRC difference between devices was similar however a larger discrepancy was demonstrated in vivo compared to in vitro. In vivo ventilation heterogeneity (VH) indices (Scond, Sacin and LCI) in asthma and in healthy people were highly variable
between different MBNW devices but did not follow the same pattern of differences as FRC. These findings are important because currently available commercial MBNW devices have become increasingly available. Differences in MBNW results between the different devices suggest these devices are not comparable. Therefore tests performed on different MBNW commercial devices cannot be used interchangeably. VH indices must be interpreted with caution and attention paid to device and software specific calculation algorithms and reference equations.

6.2 Loss of lung elastic recoil is associated with small airway dysfunction in the diffusion-dependent airways (Sacin) and airflow obstruction (FEV₁/FVC).

The patient group studied in this thesis was older non-smokers with asthma and FAO. A majority of the patients in this study already had impaired lung function, as measured by spirometry during enrolment, therefore would likely have small airway dysfunction. Chapters three and four of this thesis explores how lung function impairment in this cohort may be affected by the elastic properties of the lung tissue. Elastic properties were quantified using an exponential equation fitted to the pressure-volume curve to derive indices reflecting lung elastic recoil and compliance (10).

Small airway dysfunction was measured using the MBNW test to derive indices that provide mechanistic information about ventilation heterogeneity (VH) in the conductive (Scond) and diffusion-dependent (Sacin) airways. Following on from chapter 2, one of the commercially available MBNW devices described in this chapter was used to measure VH. Sacin, yet not Scond was associated with a loss of lung elastic recoil suggesting the elastic
properties of the lung tissue play a role in determining VH in the very peripheral or diffusion dependent airways. This novel finding supports theoretical modelling evidence proposing that heterogeneity of diffusion-dependent ventilation can arise due to the heterogeneity of cross-sectional areas of airway openings in terminal airways and the acini (11). The outer wall of the distal airways are connected to lung tissue, therefore we can infer that if the lung tissue were altered this could lead to a loss of lung elastic recoil. Consequently, the lungs ability to hold the small airways, including the terminal airways and acini, open and patent would be affected. Therefore these airways could be more prone to collapsing and premature closure thus affecting VH.

This leads on to the results in chapter four, which shows that airflow obstruction (FEV₁/FVC) was associated with increased lung compliance and airway resistance (R₅) was associated with a loss of lung elastic recoil. These results provide further support that the elastic properties of the lung tissue contribute to lung function impairment in older non-smoking asthmatics with FAO. These novel findings do not refute the role of airway remodelling in asthma however, suggests that factors other than the airways play a role in the mechanisms leading to FAO in asthma.

6.3 The ageing process contributes to fixed airflow obstruction.

Another interesting finding was the association between worse airflow obstruction (FEV₁/FVC) and increasing age. It is well known that lung function declines with age (12, 13), however as discussed in chapter 4, this association remained even after accounting for age (i.e. the association was
present using z-scores, which already accounts for age). It is also known that lung elastic recoil declines with age (14-17) and an association between loss of lung elastic recoil and increasing age was demonstrated in chapter 4. This association was no longer significant when age (i.e. using z-scores) was taken into account however there was a trend. These results suggest the ageing process is an additional factor contributing to lung function impairment and FAO in asthma.

The associations between the lungs elastic properties and Sacin, airflow obstruction and airway resistance may be related to this patient cohort being older. Functionally, older lungs can behave similarly to smokers with chronic obstructive pulmonary disease (COPD), probably because of the structural changes that occur with age (18, 19). Similarly non-smokers with asthma who are of an older age group, may demonstrate lung function abnormalities that are akin to that seen in COPD (i.e. FAO) (8). Quantifying the contribution of age is difficult to determine, however it is likely that the combination of age and asthma have a synergistic effect on lung function impairment and FAO development. The role of age on inflammation and immunosenescence in asthma and FAO also remains to be determined.

6.4 Steroid insensitive fixed airflow obstruction is not related to airway inflammation in older non-smokes with asthma.

Chapter five explores airway inflammation in older non-smokers with asthma and FAO. Variable levels of neutrophils, eosinophils and inflammatory cytokines were detected in bronchoalveolar (BAL) fluid in this small cohort. However there was a disconnect between measures of airway inflammation
and lung function (spirometry, resistance and reactance and lung elastic recoil). The lack of a relationship between lung function and inflammation measured in this cross-sectional analysis suggests persisting airway inflammation does not affect lung function following high dose ICS/LABA treatment. However, the effect of previous inflammation and inflammation over time on lung function and FAO development was not assessed, therefore is not known. A standardized period and dose of ICS/LABA treatment was used to minimize potential confounders however most patients were not steroid naïve thus the study treatment may have had minimal additional effect on the inflammatory profile. This is supported by the absence of any significant change in spirometry from enrolment and after the two-month study period. Resistance ($R_5$) did decline after two months of high dose ICS/LABA and could potentially have been related to mucus impaction.

6.5 Future directions

6.5.1 MBNW and measurement of small airway function

MBNW provides important complimentary information to currently available lung function tests however is not yet widely used in clinical practice. MBNW use is predominantly in the research setting. Its role may be more relevant in detecting disease early, before abnormalities are detected by spirometry (20-23), at which point it may be too late because the damage may be irreversible. Integrating MBNW use into routine clinical practice will help improve our understanding and management of lung disease. However, more work is needed to validate commercial MBNW equipment to ensure measurements are robust, reliable and accurate. Large multi-centre studies
comparing measurements between commercial devices in health and disease, also covering a wide age range and ethnicities, would be required. In addition, longitudinal studies using birth cohorts would assist with determining the expected change in MBNW indices over time/with age in health and disease. Other future work in the MBNW field could involve expanding on the simple syringe-based lung model described in chapter 2, to allow simulation of paediatric lung volumes. This would also require a lung model with the ability simulate breathing techniques used in paediatric testing (i.e. free breathing rather than a fixed volume of 1 L used in adult testing (24)). In addition, modifying the physical lung model to enable measurement of the more complex VH indices may help with ongoing standardization efforts.

6.5.2 Exploring mechanisms contributing to the loss of lung elastic recoil in asthma
Loss of lung elastic recoil may occur as a result of lung tissue changes (i.e. lung remodeling) (8), however needs further investigation, ideally using lung tissue. Obtaining lung tissue is a challenge due to the risks involved; hence there are very limited studies that include lung tissue assessment in living asthmatics. However less invasive tests such as imaging could be used as a substitute. Future studies could incorporate the use of high-resolution computed tomography scans to assess for lung tissue changes such as emphysema or enlarged alveoli, in addition to changes of airway remodeling that are known to occur in asthma.

The contribution of the ageing process to the loss of lung elastic recoil is difficult to disentangle from asthma itself. Either longitudinal studies in non-
smokers with asthma and/or comparing results from this thesis to a control group would be required. The control group would consist of younger non-smoking asthmatics and/or a healthy control group of a similar age range (i.e. older). Longitudinal assessment of the lungs elastic properties would be important to measure, to help determine the rate of change or decline in loss of elastic recoil. The oesophageal balloon test is also somewhat invasive and this approach may not be practical. Therefore, future studies could utilise a more practical and less invasive method of assessing the elastic properties of the lung tissue. Use of the forced oscillation technique (FOT) may be the answer. Future studies could build on previous work in COPD, which has used the FOT indices inspiratory and expiratory reactance ($X_{rs}$) to determine the presence of expiratory flow limitation (25). Using these parameters may identify those with asthma who have enhanced airway narrowing due to lung tissue alteration and subsequent loss of lung elastic recoil.

The role of inflammation and possibility of proteolysis of the lung tissue also needs further exploration. Surrogate markers of tissue turnover and degradation such as YKL-40 could be assessed in BAL, endobronchial biopsies and/or peripheral blood samples, because lung tissue samples may be more challenging to obtain. Pro-inflammatory markers that may contribute to the proteolytic cascade could also be measured, such as matrix metalloproteinases (MMPs) and neutrophil elastase. In addition, the effect of inflammation and possible proteolysis over time warrants further investigation with longitudinal studies.
6.6 Final conclusions
Asthma is a heterogeneous disease that is genetically complex and cannot be explained by one mechanism alone (26). FAO is one of many asthma phenotypes yet current tools do not readily identify those who are at risk of developing FAO or provide definitive mechanisms leading to FAO. FAO in asthma demonstrates functional similarities with COPD, however the underlying mechanisms and pathogenesis cannot be the same, as a smoking history may not be present. Airway-lung tissue inter-dependence is extremely important and the role of both the airways and lung tissue need to be accounted for, in addition to the contribution of the inflammatory pathways. Studies focusing on treatment of FAO in asthma seem far off because the mechanisms are not well understood.

Findings from this thesis highlight the importance of mechanisms other than airway remodeling that play a role in FAO in asthma. Changes to the lungs elastic properties resulting in increased compliance or reduced lung elastic recoil are suggested to make a significant contribution. ‘Lung remodeling’ is a potential pathological process leading to lung tissue changes and may be an alternate asthma paradigm to airway remodelling. This FAO phenotype tends to occur in older people with asthma thus contribution of the ageing process should not be ignored. Additionally, the underlying cellular mechanisms, which may lead to a loss of elastic recoil in asthma needs further investigation. Whether immunosenescence, asthma or both contribute to the underlying cellular mechanisms remains elusive. This may represent a potential pathway by which the FAO phenotype is manifest and highlights the need for
development of novel treatments that target ‘lung remodelling’ and loss of elastic recoil in asthma. This may involve targeting multiple rather than single inflammatory pathways because the inflammatory pathways are complex.
References


7 Appendix
In vitro and in vivo functional residual capacity comparisons between multiple-breath nitrogen washout devices

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ABSTRACT  Functional residual capacity (FRC) accuracy is essential for deriving multiple-breath nitrogen washout (MBNW) indices, and is the basis for device validation. Few studies have compared existing MBNW devices. We evaluated in vitro and in vivo FRC using two commercial MBNW devices, the Exhalyzer D (EM) and the EasyOne Pro LAB (ndd), and an in-house device (Woolcock in-house device, WIMR).

FRC measurements were performed using a novel syringe-based lung model and in adults (20 healthy and nine with asthma), followed by plethysmography (FRCpleth). The data were analysed using devicespecific software. Following the results seen with ndd, we also compared its standard clinical software (ndd v.2.00) with a recent upgrade (ndd v.2.01).

WIMR and EM fulfilled formal in vitro FRC validation recommendations (>95% of FRC within 5% of known volume). Ndd v.2.00 underestimated in vitro FRC by >20%. Reanalysis using ndd v.2.01 reduced this to 11%, with 36% of measurements ≤5%. In vivo differences from FRCpleth (mean±sd) were 4.4±13.1%, 3.3±11.8%, −20.6±11% (p<0.0001) and −10.5±10.9% (p=0.005) using WIMR, EM, ndd v.2.00 and ndd v.2.01, respectively.

Direct device comparison highlighted important differences in measurement accuracy. FRC discrepancies between devices were larger in vivo, compared to in vitro results; however, the pattern of difference was similar. These results represent progress in ongoing standardisation efforts.

Multiple-breath washout devices are not yet comparable

Introduction

Abnormalities in the small airways can be detected using the multiple-breath nitrogen washout (MBNW) test, which provides a more sensitive measure of small airway function than spirometry [1–3]. MBNW provides insightful information about the small airways, using indices that assess gas-mixing efficiency in the lung (Lung Clearance Index, LCI) and mechanistic information about ventilation heterogeneity in the conduction and diffusion-dependent airways ($S_{cond}$ and $S_{acin}$). These indices rely on accurate functional residual capacity (FRC) measurements [4].

Advances in technology have allowed the development of new commercial MBNW devices; however, there are still limited published data comparing measurements between devices. Previous studies have compared in-house and/or commercial devices against standard body plethysmography as well as mass spectrometry, often considered the gold standard [5–8]. Many of these studies have used lung models, assessed measurements in the paediatric population and used inert gases rather than $N_2$ [9–11]. Limited published data have also shown inconsistencies between lung model and adult measurements [6].

Furthermore, the effect of software upgrades in a rapidly evolving field has not been studied extensively. Previous studies in one specific device have shown that software changes can have a significant impact on results [12, 13]. Recommendations for MBNW techniques have been published in the European Respiratory Society/American Thoracic Society (ERS/ATS) Consensus statement [4]; however, ongoing work is still needed to improve the standardisation of equipment, technical specifications and algorithms for the calculation of indices.

The aim of this study was to evaluate FRC measured using two commercial MBNW devices, the Exhalyzer D (EM) from Eco Medics AG (Duernten, Switzerland) and the EasyOne Pro LAB (ndd) from ndd Medical Technologies (Zurich, Switzerland). We also examined a third, independent, previously published [2] in-house device at the Woolcock Institute of Medical Research (WIMR), which measures $N_2$ directly, in contrast to the commercial devices. FRC measurements from the devices were compared against a known volume using a syringe lung model (in vitro) and against plethysmographic lung volume in healthy and asthmatic adult subjects (in vivo). In addition, we compared analyses using two different software versions of the ndd device, because a software update involving major changes to $N_2$ calculation has recently become available. Previous data [14, 15] have shown significant FRC underestimation using the older, widely available software.

Methods

Study design

In vitro and in vivo FRC measurements were performed using the three different MBNW devices, in random order as determined by a computer-generated randomisation sequence. The study was approved by the Northern Sydney Local Health District Human Research Ethics Committee (protocol no. LNR/16/HAWKE/11). Written informed consent was obtained from all recruited participants.

The lung model

In vitro measurements were performed using an optimised syringe lung model (figure 1). A 3 L volume calibration Hans Rudolph syringe (5530 series) was modified to produce the physiological expirogram

**FIGURE 1** Physical lung model composed of a) a 3 L Hans Rudolph syringe, b) an attachment made from 18 flexible tubes, c) a helical mixer device inserted at the syringe entrance, and d) an adjustable syringe stopper.
encountered during in vivo testing. This was accomplished by incorporating an attachment on the front of the syringe consisting of 18 flexible Tygon (S3 E-3603) tubes of varying lengths (internal diameter of 0.048 cm and lengths ranging from 10 to 49.5 cm) and permeability coefficients for CO$_2$ of $360 \times 10^{-11}$ cm$^2\text{s}^{-1}\text{cmHg}^{-1}$, N$_2$ of $40 \times 10^{-11}$ cm$^2\text{s}^{-1}\text{cmHg}^{-1}$ and O$_2$ of $80 \times 10^{-11}$ cm$^2\text{s}^{-1}\text{cmHg}^{-1}$, to produce the phase I and II portions of the expirogram, and a 3D-printed helical mixer device inserted at the syringe entrance to optimise gas mixing and produce a smooth phase III.

The target FRC was calculated by adding the known syringe volume to the dead space of the lung model attachments. The syringe volume was adjusted via a stopper on the syringe plunger to predetermined positions. The dead space of the attachments was 0.310 L, determined from the computer-aided design specifications and confirmed with water displacement.

**In vitro study**

In vitro measurements were performed in triplicate on each device using four different FRC volumes (1.51 L, 1.81 L, 2.11 L and 2.31 L). A standardised adult protocol (tidal volume of 1–1.3 L) [2, 4, 16–18] was used. After at least 5 syringe strokes with a stable end expiratory volume, the washout phase was commenced and 100% oxygen was switched on. Syringe strokes were continued until end-tidal N$_2$ concentration decreased to 1/40th of the starting end-tidal N$_2$ concentration [4]. Between measurements, at least 10 strokes were first performed to expel any residual oxygen within the syringe lung model and to ensure the N$_2$ had returned to baseline. A single operator (K.O. Tonga) performed all measurements under ambient temperature and pressure dry (ATPD) conditions.

**In vivo study**

Healthy volunteers were recruited from the WIMR and defined as current non-smokers with <10 pack-years smoking history and with no history of acute respiratory illness within the preceding month. Subjects with asthma were recruited if they had a physician diagnosis of asthma: history of asthma symptoms, previously documented significant bronchodilator reversibility on spirometry and/or a positive bronchoprovocation challenge test and on inhaled bronchodilator and/or inhaled corticosteroid medication. Short acting β-agonists were withheld for 6 h and long-acting β-agonists for 24 h before testing.

All participants were over the age of 18 years and completed a standardised interview on respiratory and general health before performing pulmonary function tests (PFTs) in the following order: MBNW, spirometry and body plethysmography. All tests were performed in a single session at the Woolcock Institute of Medical Research.

Spirometry and body plethysmography were performed according to ATS/ERS Guidelines, using a BodyBox 5500 (Medisoft Corporation, Sorrines, Belgium). MBNW was performed in triplicate in the seated position, using a noseclip and device-specific bacterial filter and mouthpiece attachments. Tests were conducted according to ERS/ATS consensus statement [4], using a standardised adult protocol (tidal volume of 1–1.3 L) [2, 16–18]. After at least 5 breaths with a stable end expiratory lung volume, the washout phase was commenced and 100% oxygen was switched on. Breaths were continued until end-tidal N$_2$ concentration decreased to 1/40th of the starting end-tidal N$_2$ concentration [4]. The time interval between measurements was standardised to twice the previous washout time [4].

**MBNW hardware and software**

Details of the WIMR, EM and ndd devices are provided in the supplementary material. Briefly, the WIMR device measures N$_2$ directly via a side-stream N$_2$ analyser [2]. The commercial devices both measure N$_2$ indirectly, based on side-stream CO$_2$ and O$_2$ in the EM device and molar mass and CO$_2$ in the ndd device [6]. Daily calibration and/or verification of each device was performed as described in the supplementary material.

FRC was calculated as the net volume of N$_2$ expired divided by the difference between end-tidal N$_2$ concentration at the start and end of the washout portion of the test [4]. FRC values were corrected for the pre-capillary dead space volume for each device. MBNW data were analysed using software specific to each device. For the WIMR device, custom-written software (Solver Version 1.3.2.18) was used. For the EM device, Spiroware Version 3.1.6 was used. For the ndd device, the same measurements were analysed using two different software versions (described in the supplementary material): clinical software Version 2.00.01.05 (termed “ndd v.2.00”) and the recent upgrade released in March 2016, Version 2.01.00.09 (termed “ndd v.2.01”). The ndd v.2.01 software contains updates to the N$_2$ calculation method and improved flow-gas delay synchronisation.
Measurements were included for analyses if acceptability criteria were met. For the in vitro study, measurements were deemed acceptable if three tests were within 10% of the mean FRC [19] value across the triplicate measures. For the in vivo study, measurements were deemed acceptable if two or more tests were performed adequately according to the ERS/ATS Consensus statement [4]. Tests were excluded if there was evidence of leak during testing, the tidal volume of the first breath or more than a third of breaths during washout was outside 1–1.3 L, and if the end-tidal N₂ concentration did not reach the recommended 1/40th of the initial concentration during data acquisition.

Statistical analysis
FRC data were analysed using IBM SPSS Version 22, and graphs were generated using GraphPad Prism Version 6.0. Summary data are presented as mean±SD. The accuracy of in vitro FRC was assessed according to the consensus statement [4] (FRC values within 5% of the known volume for at least 95% of values) and expressed as absolute (L) difference (measured FRC – lung model FRC) and relative (%) difference (absolute difference×100/lung model FRC). In vivo FRC was compared with body plethysmography FRC (FRCpleth). FRC differences between devices were assessed using Bland and Altman plots with 95% limits of agreement and by non-parametric one-way repeated measures ANOVA (Friedman’s test) and post hoc tests. p-values <0.05 were considered statistically significant.

Results
In vitro comparison
A total of 108 measurements were performed across the three MBNW devices (table 1). Differences between measured and lung model FRC values are shown in figure 2 and table 2. FRC accuracy was within the specified 5% accuracy range of the lung model FRC for 100% of WIMR measurements and 97% of EM measurements. All FRC measurements using ndd v.2.00 were underestimated and not within the specified 5% accuracy range. Using ndd v.2.01, accuracy improved, although only 36% of measurements were within the 5% accuracy range.

In vivo comparison
A total of 29 subjects (20 healthy controls and 9 asthmatics) were included in the analyses, and their characteristics are outlined in table 3. The mean (±SD) FRC measured by the WIMR (3.27±0.82 L) and EM (3.56±0.92 L) devices did not differ significantly from FRCpleth (3.44±0.77 L) or from each other, and FRC measured by ndd v.2.00 (2.71±0.64 L) was significantly lower than FRCpleth, WIMR and EM (p<0.0001) (figure 3 and table 4). The same pattern of FRC differences was observed in healthy control and asthmatic subjects.

When the ndd data were reanalysed using ndd v.2.01 software, end-tidal N₂ concentrations became systematically higher, such that only 9 of the 29 subjects had measurements that reached an acceptable end-tidal N₂ concentration. However, a majority of the measurements exhibited a stable plateau at the end of the washout. For the purposes of this study, all 29 subjects were included for analysis to allow comparison of the effect on FRC. FRC increased when reanalysed using ndd v.2.01 (3.06±0.71 L) and was also significantly lower than FRCpleth (p=0.011) and the EM device (p<0.0001); however, it did not differ from the WIMR device.

Discussion
Summary of results
This is the first study to compare in vitro and in vivo FRC measurements in healthy and asthmatic adults, using two currently available commercial devices and one in-house MBNW device. The WIMR device showed the highest accuracy and reliability, with the lowest absolute and relative differences compared to the lung model FRC, and the only device that met the specifed 5% accuracy range for all measurements.

| TABLE 1 | In vitro functional residual capacity (FRC) measurements on each device |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Lung model FRC volume | Trials | WIMR | EM | ndd v.2.00 | ndd v.2.01 |
| 2.31     | 9     | 2.30±0.02 | 2.39±0.02 | 1.78±0.14 | 2.16±0.04 |
| 2.11     | 9     | 2.10±0.02 | 2.17±0.02 | 1.72±0.12 | 2.00±0.03 |
| 1.81     | 9     | 1.84±0.02 | 1.88±0.02 | 1.37±0.11 | 1.72±0.03 |
| 1.51     | 9     | 1.53±0.03 | 1.53±0.02 | 1.25±0.05 | 1.43±0.02 |

Data presented as mean±sd, unless otherwise stated. Measurements are in litres. WIMR: Woolcock in-house device; EM: Exhalyzer D device; ndd v.2.00: EasyOne Pro LAB device Version 2.00.01.05; ndd v.2.01: EasyOne Pro LAB device analysed in Version 2.01.00.09.
measured in vitro FRC closest to the known lung model volume. The mean overestimation of in vitro FRC and FRC\textsubscript{pleth} was 3% by the EM device, in comparison to a mean 21% underestimation by the ndd device. However, on reanalysis using ndd v.2.01, underestimation was reduced to 5% (in vitro) and 11% (in vivo), respectively. There were statistically significant differences in FRC measurements between commercial MBNW devices, although this difference was relatively small between EM and ndd v.2.01. Furthermore, the pattern of differences (i.e. over-estimation or under-estimation of FRC) between devices was consistent using both the in vitro physical lung model and the in vivo measurements.

### Comparison to other studies

The Consensus recommendations based on expert opinion stated that 95% of the in vitro FRC measurements should be within 5% of the target volume [4]. WIMR and EM devices fulfilled this criterion; however, the ndd device did not achieve 5% accuracy for any measurements (v.2.00). When measurements were reanalysed using ndd v.2.01, 36% fulfilled this criterion. Both commercial devices were reported previously to be highly accurate in measuring FRC using a physical lung model [14]. This lung model was water-based and incorporated BTPS (body temperature, ambient pressure, saturated) correction, whereas ours did not. Despite this, our results showed the same pattern of over/underestimation in vitro and in vivo. This is consistent with their in vivo results in 10 healthy adults, where EM overestimated FRC\textsubscript{pleth} by 14% and ndd underestimated by 23%. A recent paediatric study with healthy control and cystic fibrosis patients also reported the same pattern, i.e. higher FRC measurements using the EM device compared to the ndd device [15]. Other in vitro and in vivo paediatric studies exist [9, 11], but they involve different tracer gases, which limits comparison.

### Impact of N\textsubscript{2} estimation method

Differences in FRC across the three devices are probably attributable to device and software variations. This includes the method used to calculate N\textsubscript{2} concentration. The WIMR device measures N\textsubscript{2} directly, whereas the EM device uses Dalton’s law to compute N\textsubscript{2} by simple subtraction of other constituent gases in the expired air. These two methods were found to be similar. In contrast, the ndd v.2.00 device uses the concept of a prototype expirogram, derived from the shape of the molar mass versus expired volume curve in the early breaths of the washout. The expired N\textsubscript{2} volume for each breath is then determined by scaling

### TABLE 2 Differences in in vitro functional residual capacity (FRC) measurements from the lung model functional residual capacity for four different lung volumes

<table>
<thead>
<tr>
<th>Lung model FRC volume</th>
<th>Subjects</th>
<th>WIMR Absolute</th>
<th>WIMR Relative</th>
<th>EM Absolute</th>
<th>EM Relative</th>
<th>ndd v.2.00 Absolute</th>
<th>ndd v.2.00 Relative</th>
<th>ndd v.2.01 Absolute</th>
<th>ndd v.2.01 Relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.31</td>
<td>9</td>
<td>−0.01±0.02</td>
<td>−0.2±0.8%</td>
<td>0.07±0.02</td>
<td>3.3±1.0%</td>
<td>−0.54±0.14</td>
<td>−23.4±6.1%</td>
<td>−0.15±0.04</td>
<td>−6.3±1.7%</td>
</tr>
<tr>
<td>2.11</td>
<td>9</td>
<td>−0.01±0.02</td>
<td>−0.4±0.9%</td>
<td>0.06±0.02</td>
<td>2.9±0.9%</td>
<td>−0.39±0.12</td>
<td>−18.5±5.7%</td>
<td>−0.11±0.03</td>
<td>−5.2±1.5%</td>
</tr>
<tr>
<td>1.81</td>
<td>9</td>
<td>0.03±0.02</td>
<td>1.6±1.4%</td>
<td>0.07±0.02</td>
<td>3.7±1.0%</td>
<td>−0.44±0.11</td>
<td>−24.1±6.4%</td>
<td>−0.09±0.03</td>
<td>−4.7±1.6%</td>
</tr>
<tr>
<td>1.51</td>
<td>9</td>
<td>0.02±0.03</td>
<td>1.7±13.1%</td>
<td>0.02±0.02</td>
<td>1.1±1.3%</td>
<td>−0.26±0.05</td>
<td>−17.1±3.0%</td>
<td>−0.08±0.02</td>
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</table>

Data presented as mean±sd, unless otherwise stated. Absolute measurements are in litres. WIMR: Woolcock in-house device; EM: Exhalyzer D device; ndd v.2.00: EasyOne Pro LAB device Version 2.00.01.05; ndd v.2.01: EasyOne Pro LAB device analysed in Version 2.01.00.09.
the prototype expirogram to match the end-expiratory N₂ concentration for that breath. Potential inaccuracies could occur if the expirogram shape changed greatly during the course of the washout, as is typically observed in more severe obstructive airways disease. However, ndd v.2.01 uses a combination of molar mass measurements and Dalton’s law to compute N₂, on a point-by-point basis for the entire expirogram. This could partly explain the improved accuracy of ndd v.2.01 and the reduced FRC discrepancy between the new software and the WIMR and EM devices.

**Impact of other software changes**

The other major change in ndd v.2.01 involve modifications to the estimation of delay between the flow and respective gas measurement points, which are more robust to variation in breathing patterns, particularly very brief pauses in flow (personal communication with the manufacturer). Differences in delay time potentially have a large effect on FRC calculations [20, 21].

Newer software versions were available at the time of writing (v.2.2.0.15 onwards). These involve major changes to the user interface; however, they fundamentally employ the same indirect N₂-based FRC calculation and delay estimation method. Our results thus have significant implications on the reanalysis of old data and are relevant to ndd v.2.01 as well as any newer versions. Furthermore, they illustrate the importance of analysis methods and any preprocessing algorithms that may be used, as well as the need for software transparency [22]. They also highlight the importance of ongoing validation and that standardisation efforts are gradually working.

It should be noted that this study was unable to confirm whether collecting data using ndd v.2.01 would have further improved the FRC accuracy for the ndd device. A large proportion of the in vivo tests reanalysed using the new software failed to meet end-of-test criteria, possibly due to the underestimation

### TABLE 3 Subject demographics and standard lung function measurements

<table>
<thead>
<tr>
<th></th>
<th>All subjects</th>
<th>Controls</th>
<th>Asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects n</td>
<td>29</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Males/females n</td>
<td>12/17</td>
<td>8/12</td>
<td>4/5</td>
</tr>
<tr>
<td>Age years</td>
<td>37±12</td>
<td>36±12</td>
<td>37±13</td>
</tr>
<tr>
<td>Height cm</td>
<td>172±12</td>
<td>174±12.5</td>
<td>168±10</td>
</tr>
<tr>
<td>BMI kg·m⁻²</td>
<td>24.1±3.63</td>
<td>23.9±3.04</td>
<td>24.6±4.10</td>
</tr>
<tr>
<td>Smoking history pack-years</td>
<td>2.5±3.2</td>
<td>2.75±3.31</td>
<td>0.70±0.00</td>
</tr>
<tr>
<td>FEV₁ L</td>
<td>3.66±0.93</td>
<td>3.75±0.93</td>
<td>3.47±0.95</td>
</tr>
<tr>
<td>FVC L</td>
<td>4.75±1.17</td>
<td>4.77±1.16</td>
<td>4.71±1.25</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.77±0.06</td>
<td>0.79±0.05</td>
<td>0.74±0.08</td>
</tr>
<tr>
<td>TLC L</td>
<td>6.51±1.38</td>
<td>6.55±1.41</td>
<td>6.62±1.41</td>
</tr>
<tr>
<td>FRC&lt;sub&gt;pleth&lt;/sub&gt; L</td>
<td>3.44±0.77</td>
<td>3.49±0.78</td>
<td>3.34±0.80</td>
</tr>
</tbody>
</table>

Data presented as mean±SD unless otherwise stated. BMI: body mass index; FEV₁: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; FRC<sub>pleth</sub>: functional residual capacity measured using body plethysmography.

FIGURE 3 Bland–Altman plots of in vivo functional residual capacity (FRC) measurements on each device. Data are plotted as body plethysmography FRC (FRC<sub>pleth</sub>) minus multiple-breath nitrogen washout (MBNW) FRC, expressed as the absolute difference versus mean of FRC<sub>pleth</sub> and MBNW FRC. Absolute differences (circles), mean difference and upper and lower limits of agreement (mean difference±SD of differences) are shown as dashed lines. a) Woolcock in-house device; b) Exhalyzer D device; c) EasyOne Pro LAB device analysed in Version 2.01.00.09.

https://doi.org/10.1183/23120541.00011-2017
TABLE 4 Differences in in vivo functional residual capacity (FRC) measurements from body plethysmography FRC on each device

<table>
<thead>
<tr>
<th>Subjects n</th>
<th>WIMR</th>
<th>EM</th>
<th>ndd v2.00</th>
<th>ndd v2.01</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute</td>
<td>Relative</td>
<td>Absolute</td>
<td>Relative</td>
</tr>
<tr>
<td>Controls</td>
<td>-0.13±0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.3±13.0%</td>
<td>0.17±0.41</td>
<td>4.7±11.8%</td>
</tr>
<tr>
<td>Asthmatics</td>
<td>-0.24±0.45</td>
<td>-6.8±13.8%</td>
<td>0.01±0.39</td>
<td>0.12±12.0%</td>
</tr>
<tr>
<td>All subjects</td>
<td>-0.17±0.45</td>
<td>-4.4±13.1%</td>
<td>0.12±0.40</td>
<td>3.3±11.8%</td>
</tr>
</tbody>
</table>

Data presented as mean±sd, unless otherwise stated. Absolute measurements are in litres. WIMR: Woolcock in-house device; EM: Exhalyzer D device; ndd v.2.00: EasyOne Pro LAB device Version 2.00.01.05; ndd v.2.01: EasyOne Pro LAB device analysed in Version 2.01.00.09. <sup>a</sup>: missing data for one subject.

of N<sub>2</sub> in the old software. One could speculate that if acceptable end-tidal N<sub>2</sub> concentration had been met, in vitro FRC may have been slightly closer to the true lung volume and in vivo FRC closer to FRC<sub>pleth</sub>.

**Impact of patient factors**

Other factors that may affect FRC measurements include the effect of breathing patterns during the washout. Different mouthpieces, resistances, the nature of the real-time displays of volume and patient breathing incentives, and even different open bypass systems delivering oxygen [15], may have affected breathing patterns. However, dead space correction was applied in each device and the same 1 L breathing protocol was used. More importantly, the direction of over/underestimation was preserved regardless of in vitro and in vivo FRC comparisons, i.e. independent of BTPS correction, breathing pattern and the presence of CO<sub>2</sub>. Thus, it is unlikely that patient factors contributed significantly to the differences between devices. In addition, the differences between MBNW FRC and FRC<sub>pleth</sub> on each device were of similar magnitude between healthy controls and subjects with asthma with well-preserved spirometry. It is unknown whether the same results would hold in subjects with more airway obstruction, or other patient groups such as chronic obstructive pulmonary disease (COPD).

**Limitations**

Our study had a number of limitations. First, we compared differences only in FRC between devices; however, LCI, S<sub>acin</sub> and S<sub>cond</sub> are the more clinically relevant MBNW indices. There are no known lung models to evaluate these other indices and a formal comparison of these indices in vivo was not performed. Nevertheless, high-quality FRC measurement is necessary for evaluation of these indices. Second, between-session variability for FRC has not been defined, so it is not known whether differences between in vivo measurements are within the limits of normal test variability. Third, the applicability of our results to the paediatric population is unknown. However, the simplistic design of the lung model could be easily adapted to a smaller syringe. Also, we only evaluated the standardised 1 L breathing protocol [23], so the effect of the free breathing protocol used by other groups [24], and especially in infants and children, is unknown. Furthermore, the syringe is an ATPD model rather than a BTPS model, which is less representative of the actual physiological situation but more practical for routine laboratory use. Despite this, the same pattern of accuracy was seen in vitro and in vivo, suggesting that incorporation of BTPS correction has a minimal effect on the relative errors reported. Finally, as discussed above, we did not evaluate the accuracy of data collected using the ndd v.2.01, which is scope for further work.

**Conclusion**

We have shown differences in the measurements of FRC between three MBNW devices in a physical syringe model of the lung. The syringe lung model used in this study was simple, portable and relatively easy to produce compared to that used in other studies [6, 25, 26]. It would allow a simple and practical way to calibrate or check the MBNW setup, because it tests the combined accuracy of volume and N<sub>2</sub> concentration measurement during a more realistic expirogram and the correct alignment of these signals. The use of such a syringe model may be beneficial for comparison between devices and laboratories and for quality assurance monitoring. FRC differences were also reflected in vivo in healthy and asthmatic subjects, in relation to plethysmographic FRC. While further work is required to improve accuracy, the in vivo differences observed are small and probably not clinically significant. Differences are likely to reflect the method of calculating N<sub>2</sub> concentration and other software factors. How these FRC errors translate to the accuracy of other indices has been explored for LCI [15], but not S<sub>acin</sub> and S<sub>cond</sub> so far. Nevertheless, our results show that the state of the art is closer to achieving better comparability and standardisation for FRC accuracy across existing MBNW devices.
Acknowledgements
The authors acknowledge the contribution of Gunnar Unger, Martin Turner, Aaron Skelsey and Sunny Ye, who were involved with the development of the syringe lung model. Both manufacturers for the Eco Medics and ndd devices were consulted for the technical accuracy of the manuscript. The authors acknowledge the contribution of Christian Buess, who provided additional technical information regarding the ndd software where indicated in the manuscript. Neither manufacturer influenced the study design, results or interpretation.

References

https://doi.org/10.1183/23120541.00011-2017
In vitro and in vivo functional residual capacity comparisons between multiple breath nitrogen washout devices.

Supplementary material

Description of MBNW devices:

Custom built in-house device (WIMR)

WIMR measures flow via a standard mesh-type pneumotachograph (Hans Rudolph® Inc, Shawnee, KS, USA, model no. 3700) and pressure transducer (Sensortechnics GmbH, Puchheim, Germany, model no. HCLA02X5EB) (1). N\textsubscript{2} is measured directly via a side-stream N\textsubscript{2} analyser (Medgraphics Corporation, St Paul, MN, USA, model no. 762033-001). A fixed orifice valve (Bird Precision, Waltham, MA, USA, Ruby type model no. RB82453) was incorporated which served to improve the linearity response of the N\textsubscript{2} analyser. The pre-capillary dead space of the set up, which comprised the mouthpiece (Jaeger silicon adult size), bacterial filter (Respigard II 303E), fixed orifice valve attachment and the pneumotachograph flowhead, was 35 ml. The post-capillary deadspace was 17.67 ml.

Daily calibration and verification of gas and flow signals was performed as per a standardised protocol for the in-house device (1). Flow verification was performed using a 1-L Hans Rudolph® calibration syringe connected to the flow head via a standard bacterial filter. Verification was performed over five full syringe strokes and measured within ± 3% accuracy, respectively. A two-point N\textsubscript{2} calibration was performed by blocking the expiratory port in order to direct all bias flow gas to the N\textsubscript{2} analyser, with room air and 100% O\textsubscript{2} gas as reference points.
A fixed delay of 50 ms was used to align the flow and N₂ channels. This delay was calculated by injecting a bolus of 100% O₂ gas using a syringe attached to the entrance of the fixed orifice valve (i.e. the gas measurement point), resulting in a step increase in N₂. The delay was then defined as the difference between time at 50% of the step change in flow and 50% of the step change in N₂.

ECO MEDICS AG Exhalyzer® D device (EM)

EM measures N₂ indirectly via a main-stream infra-red CO₂ sensor (CapnostatH 5®, Respironics Novametrix LLC, Wallingford, CT, USA) and a side-stream laser oxygen sensor (Oxigraf, Inc, Mountain View, CA, USA) (2). Since the concentrations of both O₂ and CO₂ are obtained, the concentration of N₂ is then simply calculated using Dalton’s law (see Equation 1).

\[ f_{N_2} + f_{O_2} + f_{CO_2} + f_{H_2O} + f_{Ar} = 1 \] (Equation 1)

with assumptions regarding the partial pressures of water vapour and Argon (2). The pre-capillary deadspace of the setup, which comprised the mouthpiece (VacuMed thermoplastic adult size), bacterial filter (Respigard II 303E), and half the internal volume of the ultrasonic flowmeter with a dead space reducer insert (adult size #3), was 58 ml. The post-capillary deadspace was 22 ml.

Calibration of flow and gas channels were performed daily, as outlined below. Flow was calibrated as per manufacturer’s guidelines for the adult set up, using a 1-L Hans Rudolph® calibration syringe connected to the flow head via
a standard bacterial filter. Ten full syringe strokes were performed and measured within ± 3% accuracy. Two-point gas channel calibration of the O₂ sensor and zero calibration of the CO₂ sensor were conducted as per manufacturer’s guidelines and as previously described (2).

Synchronization of the flow and gas channels was performed monthly, again as previous described (2). The delays between the flow and respective gas channels were calculated and verified based on the 50% rise time in the step response in both gases seen when post-capillary dead space was reinspired, and averaged over 10 breaths in a typical human subject. These delays were used to synchronise the gas channels to the flow channel.

**ndd EasyOne Pro® LAB device (ndd)**

The ndd measures N₂ indirectly via a side-stream ultrasonic transducer for sampling of side-stream molar mass (MMss), with an additional infra-red carbon dioxide (CO₂) analyser (ndd Medizintechnik AG, Switzerland). The pre-capillary deadspace of the setup, which comprised the spirette, FRC barriette, and half the internal volume of the ultrasonic flowmeter, was 24.75 ml. The post-capillary deadspace was 15.85 ml.

The previous clinical software (“ndd old”) used the concept of a prototype expirogram, derived from the shape of the molar mass versus expired volume curve in the early breaths of the washout (3). The expired N₂ volume for each breath is then determined by scaling the prototype expirogram to match the
end-expiratory N2 concentration for that breath, and integrating the flow with the scaled expirogram.

In the updated software (“ndd new”), the N2 concentration is determined by solving Dalton’s law (Equation 1 above) and Equation 2 below simultaneously for the respective fraction of N2 and O2, i.e. fn2 and fO2:

\[
f_{N2} \cdot \text{MM}_{N2} + f_{O2} \cdot \text{MM}_{O2} + f_{CO2} \cdot \text{MM}_{CO2} + f_{H2O} \cdot \text{MM}_{H2O} + f_{Ar} \cdot \text{MM}_{Ar} = \text{MM} \quad \text{(Equation 2)}
\]

where MM is the molar mass measured in the side stream as all other quantities are either measured (fCO2 and fH2O), known (molar masses of all the gas constituents, MMxx), or assumed (fAr). The fraction of Argon is assumed to be related to the N2 concentration (fAr = 0.0093 * fn2/0.7809) as it is not absorbed by the body. Two Nafion sampling tubes are used to ensure the gas in the molar mass sensor has the same humidity as ambient.

The molar mass sensor was two-point calibrated automatically by software using room air and 100% O2 as reference points. CO2 gas sensor calibration was conducted automatically using a single point reference point, i.e. room air, prior to each trial. The calibration was manually verified prior to each testing session as follows. Flow verification was performed using a 1-L Hans Rudolph® calibration syringe connected to the flow sensor via the manufacturer’s calibration adapter. One full inspiratory pump stroke was performed followed by an expiratory pump stroke at moderate speed. Flow verification was completed after three full trials were performed and measured within ± 3% accuracy.
The new software also introduces changes to the estimation of delay between the flow and gas measurement points. In NDD old, delay was calculated automatically from the time-based cross correlation between the mainstream and sidestream molar mass signals, at the start of each trial. In contrast, NDD new uses a volume-based cross correlation method to estimate the delay, which are more robust to variation in breathing patterns particularly very brief pauses in flow (personal communication with the manufacturer).
References


Lung elastic recoil and ventilation heterogeneity of diffusion-dependent airways in older people with asthma and fixed airflow obstruction

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Loss of lung elastic recoil is associated with increased ventilation heterogeneity in diffusion-dependent airways in older nonsmokers with asthma and fixed airflow obstruction http://ow.ly/g2Sk30nbVXv


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To the Editor:

Small airways are abnormal in asthma [1]. One measurement of small airway function is $S_{acim}$, derived from the multiple-breath nitrogen washout (MBNW) test. $S_{acim}$ reflects ventilation heterogeneity in diffusion-dependent airways, and is correlated with airway hyperresponsiveness [2] and asthma control [3]. Theoretically, heterogeneity of diffusion-dependent ventilation can arise due to the heterogeneity of cross-sectional areas of airway openings in terminal airways and the acini [4]. Therefore, $S_{acim}$ may be affected by structural changes in those airways. The elastic properties of the lung may also affect $S_{acim}$, as the phase III slope, a marker of ventilation heterogeneity derived from the single-breath nitrogen washout, correlates with lung compliance in explanted lungs of smokers and in healthy lungs [5].
Steroid insensitive fixed airflow obstruction is not related to airway inflammation in older non-smokers with asthma

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Abstract

There is limited evidence linking airway inflammation and lung function impairment in older non-smoking asthmatics with fixed airflow obstruction (FAO), which can develop despite treatment with inhaled corticosteroids (ICS). We assessed lung function (spirometry, forced oscillation technique (FOT)), lung elastic recoil and airway inflammation using bronchoalveolar lavage (BAL) in non-smoking adult asthmatics with FAO, following 2 months treatment with high-dose ICS/long-acting beta-agonist. Subjects demonstrated moderate FAO, abnormal FOT indices and loss of lung elastic recoil. This cross-sectional study showed a lack of a relationship between BAL neutrophils, eosinophils, inflammatory cytokines and lung function impairment. Other inflammatory pathways or the effect of inflammation on lung function over time may explain FAO development.

Keywords: Fixed airflow obstruction, Asthma, Reduced lung elastic recoil, Airway inflammation

Irreversible or fixed airflow obstruction (FAO) can develop in long-standing asthma despite no or minimal smoking history and is associated with moderate to severe disease [1, 2]. The mechanisms of FAO in asthma are poorly understood; therefore prevention and treatment remain a challenge. Inhaled and/or oral corticosteroids improve lung function and reduce exacerbations, yet may not necessarily prevent FAO from occurring [3, 4]. Asthma severity [1, 5] and FAO development may be attributed to corticosteroid resistance or insensitivity resulting in persistent airway inflammation [6] and structural airway changes. Both eosinophilic and neutrophilic inflammation may be associated with lung function impairment and FAO in asthma, however evidence is limited and contradictory [4, 7].

In this prospective study, we investigated whether lung function impairment in older non-smokers with long-standing asthma and FAO is associated with the airway inflammation which remains after treatment with maximal dose inhaled corticosteroid (ICS). We hypothesized that the degree of lung function abnormalities would positively correlate with persistent airway inflammation in patients with asthma and FAO, thereby providing a potential mechanism for the development of FAO and its apparent steroid insensitivity.

Patients were > 40 years old, non-smokers or had a negligible smoking history with a respiratory physician diagnosis of asthma. All patients were treated with a standardized maximal dose of ICS/long-acting beta-agonist (ICS/LABA) using fluticasone/eformoterol 250 μg/10 μg metered dose inhaler via a holding chamber, two puffs twice daily, if not already taking this treatment. A baseline test skin prick test to common allergens was performed to assess atopic status. During enrolment and after 2 months of treatment, patients completed a symptom questionnaire (Asthma Control Questionnaire, ACQ-5) and performed pre-bronchodilator lung function measurements. Measurements included spirometry and the forced oscillation technique (FOT) to derive airway resistance (R50) and reactance (X50) at 5 Hz. After 2 months of treatment an oesophageal balloon was used to derive the pressure-volume (P-V) curve to assess the elastic recoil properties of the lung via the indices K, reflecting lung...
compliance, and B/A, reflecting lung elastic recoil [8]. FAO was assessed following 1 month of treatment and was defined as a < 200 ml and <12% change in spirometry post-bronchodilator (400mcg inhaled salbutamol). ICS/LABA medication was withheld for at least 24 h and short acting beta-agonist medication for at least 6 h prior to testing.

Following 2 months of ICS/LABA treatment and within a week of the lung function measurements, patients then underwent bronchoscopy with bronchoalveolar lavage (BAL) from the right middle lobe [9]. Neutrophil and eosinophil counts were obtained from BAL samples as previously described [10]. Cytokines including IL-1b, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L and TNF-α were measured in BAL supernatant using a multiplex immunoassay (Bio-Rad® Bio-Plex Multiplex Immunoassay). Univariate correlations (Spearman rank test) between lung function indices (using z-scores) and BAL samples (using raw values) after 2 months of treatment were assessed.

Nineteen patients were recruited (11 male; mean ± SD age 63 ± 9 years, asthma duration 38 ± 22 years, height 1.69 ± 0.10 m, body mass index 28.4 ± 5.8 kg/metre²); 18 completed the study. Five patients were ex-smokers with 2.2 ± 2.5 pack-years smoking history and 14/19 patients were atopic. Patients were symptomatic (ACQ-5 1.03 ± 0.92) despite taking regular asthma medications prior to enrolment (ICS 18/19; with LABA 18/19; ICS/LABA/long-acting muscarinic antagonist 5/19). One patient was on long-term low dose oral corticosteroids (Prednisone dose 5 mg) for treatment of rheumatoid arthritis.

Post-bronchodilator spirometry after 1 month of treatment showed moderate FAO (mean ± SD z-score: FEV₁ – 2.05 ± 0.75, FVC -0.61 ± 0.95, FEV₁/FVC -2.46 ± 0.90). After 2 months of treatment FOT indices were abnormal: R₅ (median (IQR) z-score: 2.7(1.8–3.2)) and X₅ (z-score: –3.9(-7.3 - -2.0)). Spirometry did not change between enrolment and after 2 months of treatment, however R₅ worsened. Eighteen patients performed lung elastic recoil measurements (median (IQR) z-score: K 1.57(−1.08–3.43) and B/A -1.18(−1.65–0.02)). Increased compliance was demonstrated in 9/18 patients (K z-score ≥ 1.64) and loss of elastic recoil in 5/18 (B/A% z-score ≤ – 1.64).

Eighteen patients performed bronchoscopy and BAL neutrophil and eosinophil cell counts were obtained in 10 patients (mean ± SD: neutrophils 9.1 ± 18.1% and eosinophils 1.9 ± 1.6%). No patients had evidence of neutrophilic airway inflammation whilst 4/10 patients had eosinophilic airway inflammation. BAL cytokines were obtained in 17 patients and results are shown in Fig. 1. BMI, spirometry, FOT and elastic recoil indices did not correlate with BAL neutrophil or eosinophil count and inflammatory cytokines (Fig. 2). Occasionally, statistically significant correlations were observed however these were the result of a single outlier.

**Fig. 1** Cytokine levels measured in bronchoalveolar lavage fluid from each patient. Each row represents a patient and each column represents different cytokines. Red indicates highest levels and bright green lowest levels. IL = interleukin, IFN-g = interferon gamma, sCD40L = soluble CD40 ligand, TNF-a = tissue necrosis factor alpha
Variable levels of neutrophils, eosinophils and cytokines were detected in this small cohort however there was a disconnect between measures of airway inflammation and lung function. The lack of a relationship in this cohort suggests persisting airway inflammation does not affect lung function following ICS treatment. However, the effect of previous inflammation and inflammation over time on lung function and FAO development remains unknown. A standardized period and dose of ICS treatment was used to minimize potential confounders however most patients were not steroid naïve thus the study treatment may have had minimal additional effect on the inflammatory profile. This is supported by the absence of any significant change in spirometry from enrolment and after the two-month study period. Adherence to study treatment was assessed after 1 month and at the two-month mark to ensure non-adherence did not play a role.

Somewhat surprisingly and in contrast to previous studies [11], this older cohort did not demonstrate neutrophilic airway inflammation although small subject numbers are a limiting factor. Furthermore, neutrophil activation was not measured and may have been increased due to inhibition of neutrophil apoptosis by inhaled corticosteroids. Despite treatment with high-dose ICS/LABA, eosinophilic inflammation persisted in a few patients and cytokines were still detectable, suggesting a steroid unresponsive inflammatory pathway. The fact that FAO develops despite treatment suggests inhaled corticosteroids may have minimal effect on airway remodeling in older people with asthma. Instead FAO in this cohort may predominantly be due to other mechanisms such as the loss of elastic recoil observed in this study, which in turn may occur as a result of lung tissue changes (i.e. lung remodeling) [2]. Lung tissue changes could be due to proteolytic enzymes disrupting lung parenchyma-terminal bronchiole attachments [12]. Inflammation in the lung tissue cannot be ignored, however our study lacks the ability to assess this. Less invasive tests such as a computer tomography (CT) scan to assess for possible lung tissue changes like emphysema [13] was also not done. A recent study demonstrated micro-emphysema, only on microscopic examination of post-mortem asthmatic lungs, which was not evident on CT imaging [2], therefore inclusion of CT imaging may not have be adequate to demonstrate lung tissue changes in our study.

**Conclusion**

In summary, this exploratory cross-sectional study has shown a lack of relationship between persistent airway inflammation and lung function impairment following a short period of maximal ICS treatment. However, other cellular mechanisms, lung tissue inflammation and the potential longitudinal effect of inflammation over time in the development of FAO in asthma warrant further investigation.

**Abbreviations**

ACQ: Asthma control questionnaire; B/A%: Reflects lung elastic recoil; BAL: Bronchoalveolar lavage; FAO: Fixed airflow obstruction; FEV$_1$: Forced expiratory volume in 1 s; FOT: Forced oscillation technique; FVC: Forced vital capacity; ICS: Inhaled corticosteroid; IFN-γ: Interferon-γ; IL: Interleukin; K: Reflects lung compliance; LABA: Long acting-beta-agonist; P-V: Pressure-volume; R$_5$: Resistance at 5 Hz; scD40L: Soluble cD40 ligand; TNF-α: Tissue necrosis factor-α; X$_5$: Reactance at 5 Hz

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**Availability of data and materials**

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

KOT, GGK and BGO contributed to conception and design of the study, analysis and interpretation of data and preparation of the manuscript. KOT, FST, JS and PS contributed to data collection and analysis. CSF, CT, PS and DGC contributed to interpretation of data and preparation of the manuscript. All authors have read and approved the final manuscript.
Ethics approval and consent to participate
The study was approved by the Sydney Local Health District Human Research Ethics Committee - CRGH (protocol no. HREC/14/CRGH/75). Written informed consent was obtained from all recruited patients.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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