UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Endobronchial ultrasound and the assessment of airway wall thickness in asthma

by

Timothy John Shaw MA, MB, BChir, MRCP

Thesis for the degree of Doctorate of Medicine

August 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF MEDICINE

Doctor of Medicine

ENDOBRONCHIAL ULTRASOUND AND THE ASSESSMENT OF AIRWAY WALL THICKNESS IN ASTHMA

by Timothy John Shaw

Post-mortem studies in asthma identify airway wall thickening involving all layers. Mathematical modelling suggests such thickening can theoretically account for the bronchial hyperresponsiveness (BHR) observed in this airway disorder, by exaggerating luminal narrowing associated with a given degree of bronchial smooth muscle constriction. Although endobronchial biopsies have provided much information about inflammatory events, they are of insufficient depth to assess whole airway thickness *in vivo*. Whilst high resolution computer tomography (HRCT) scanning lends support to the notion that the airways are thickened in asthma, this technique is limited by its sensitivity of measurement, inter-observer variability and necessity for radiation exposure. This is the first work to describe endobronchial ultrasound (EBUS) using a radial probe inserted under direct vision into the airways during fibre-optic bronchoscopy to measure total airway wall thickness (AWT) *in vivo*. The technique was validated in an *in vitro* model (sheep airway) and *in vivo* by comparison with HRCT measurements in mild/moderate asthmatic and healthy volunteer subjects. In the asthmatic group these findings were related to PC_{20} histamine, as a measure of BHR, post-bronchodilator FEV_1, reversibility to β_2 agonists, and duration of asthma. Endobronchial biopsies were assessed for histological markers of airway remodelling - reticular basement membrane thickness (RbMt), submucosal collagen and proteoglycan deposition. Inter and intraobserver variability using EBUS to measure AWT were good and were in agreement with those by HRCT. Increased AWT was found in asthmatics but contrary to that predicted by mathematical models it was inversely correlated with BHR and reversibility to β_2 agonists. Although RbMt, submucosal collagens I, III, V and proteoglycans perlican, biglycan, decorin, fibronectin were increased in asthmatic subjects over controls, no correlations were found with AWT. I suggest that increased AWT in asthmatics leads to 'stiffening' of the airways which opposes excessive bronchoconstriction and is therefore beneficial in protecting the airways from closure.

List of Contents

List of Figures

List of Tables

Acknowledgements

I wish to acknowledge the contributions of the following for their role in the studies described in this thesis:-

- All the subjects who generously gave their time and support
- My research nurses Jodie Sones and Lorraine Hewitt for assistance with subject recruitment, assessment and bronchoscopy procedures
- Dr Paul Beckett who performed 4 of the EBUS bronchoscopies
- Dr Charles Peebles for advice and support with the CT scanning
- Dr Hans Michael Haitchi for acting as my second doctor for the majority of bronchoscopy procedures
- Dr Susan Wilson and the staff of the Histochemistry Research Unitfor teaching me how to cut and stain specimens for immunohistochemistry
- Ron Lee for processing, cutting and staining the paraffin sections in chapter 3
- Helen Rigden and Janet Underwood for processing the majority of the bronchial biopsies into GMA resin as described in chapter 6
- Dr Suzie Wakely who acted as an independent observer in the repeatability experiments on ultrasound image analysis described in chapter 4
- Jas Barley in the Foetal Origins of Adult Diseases research group, University of Southampton, for provision of the animal tissue used in the explant study described in chapter 3.
- The staff of The Wellcome Trust Clinical Research Unit, Southampton General Hospital, for provided the bronchoscopy facilities and nursing time

This work would not have been possible without the support and encouragement of my supervisors Dr Peter Howarth and Dr Susan Wilson.

Finally my long suffering wife Katrina.

List of Abbreviations

ADAM	a disintegrin and metalloprotease domain
AEC	chromogen, 3 amino 9 ethyl-carbazole
ASM	airway smooth muscle
ATS	American Thoracic Society
AWT	airway wall thickness
BAL	broncho-alveolar lavage
BDP	beclomethasone diproprionate
BHR	bronchial hyperresponsiveness
BSA	body surface area
BTS	British Thoracic Society
COPD	chronic obstructive pulmonary disease
COV	covariance
CT	computed tomography
DAB	chromogen, diamino benzidine
DICOM	digital imaging and communications in medicine
EBUS	endobronchial ultrasound
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
FEV_1	forced expiratory volume in 1 second
FGF	fibroblastic growth factor
FP	fluticasone propionate
GINA	Global initiative in asthma
GMA	glycol methacrylate resin
GM-CSF	granulocyte macrophage colony stimulating factor
H&E	haematoxylin and eosin
HU	Hounsfield units

HRCT	high resolution computed tomography
ICC	intraclass correlation coefficient
ICS	inhaled corticosteroids
Ig	immunoglobulin
IGF	insulin like growth factor
IFN	interferon
IL	interleukin
MMP	matrix metalloproteinase
NO	nitric oxide
OA	ovalbumin
PC_{20}	pulmonary concentration causing 20% fall in FEV_1
PD_{20}	pulmonary dose causing 20% fall in FEV_1
PDGF	platelet derived growth factor
PEF	peak expiratory flow rate
RbM	reticular basement membrane
ROW	reverse osmosis water
SD	standard deviation
SMA	smooth muscle actin
SONAR	sound navigation and ranging
TBS	tris buffered saline
T/D	thickness to diameter ratio
TGF	transforming growth factor
Th	T-helper cell
TIF	tagged image file format
TIMP	tissue inhibitor of matrix metaloproteinases
TLCO	transfer coefficient for carbon monoxide
WA	wall area
WT	wall thickness

Chapter 1: Introduction

1 Introduction

1.1 A definition of asthma

The definition of asthma has developed over time as our understanding of the underlying processes has evolved. Initial attempts concentrated on the impact on lung function – enhanced variability in airway obstruction when compared with non asthmatics. (1, 2). The current definition from the Global INitiative in Asthma (GINA) report describes asthma as "... a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment." (3). This definition emphasises the importance of the underlying cellular and biochemical changes underlying the disease, that is to say airway inflammation and its subsequent effects on symptoms. The definition still has limitations since a proportion of asthmatics exhibit poor reversibility and some patients with COPD, but not asthma, are able to demonstrate a degree of reversibility in their airflow obstruction. The definition incorporates a suggestion that reversibility is not guaranteed though does not explicitly describe the relevance of structural changes or remodelling in the airways of asthmatic subjects that has provoked much research interest in recent years (4-15).

1.2 Bronchial hyperresponsiveness

1.2.1 Definition

Fundamental to the definition of asthma given above is the concept of bronchial hyperresponsiveness (BHR). This tendency for enhanced airway narrowing in response to allergenic and non-allergenic stimuli translates clinically into the variable shortness of breath and wheeze experienced by patients with asthma and is central to most definitions of asthma. Despite this the diagnostic relevance of BHR is still unclear since the presence of BHR is neither sensitive nor specific for asthma. In the general population prevalence figures from 4 to 35% have been described (16). It is present in almost two thirds of smokers with COPD (17) and is seen in other inflammatory conditions of the airways such as cystic fibrosis (18), bronchiectasis (19) and Sjogren's syndrome (20).

1.2.2 Historical perspective

Alexander and Paddock observed in 1921 that bronchoconstriction occurred more readily in asthmatic subjects over non asthmatics after subcutaneous injection of pilocarpine (21). Their findings were confirmed by Weiss *et al* in 1932, who reported a reduction in vital capacity in asthmatics, but not controls, after intravenous histamine (22). Tiffeneau and Beauvallet were the first to use an inhalational challenge (acetylcholine) to cause bronchonstriction (23). Curry reported that bronchonstriction could be provoked in asthmatic subjects after histamine was given intramuscularly, intravenously or by nebulisation (24).

1.2.3 Measurement

Various non specific bronchoconstrictor stimuli have been described and these fall into two groups. The first type act directly on airway smooth muscle and includes histamine, methacholine, prostaglandin D_2 and the cysteinyl-leukotrienes. The second type act indirectly by causing constrictor mediator release from cells within the airways and include adenosine, cold air, exercise and hypertonic saline (25). The former have the advantage in clinical and research practice in that the inhaled dose can be titrated upwards until a 20% fall from baseline in the forced expiratory volume in one second (FEV_1) is reached thereby giving a measure of the concentration (PC_{20}) or dose (PD_{20}) required to reach this standardised endpoint (26, 27). Histamine and methacholine have a relatively short-lived bronchoconstrictor action of 1-2 hours and the effects can be reversed by inhalation of β_2 agonist medication. The inflammatory infiltrate and mediator release into the airways which characterises the late bronchoconstrictor response seen at 4 – 8 hours after allergen challenge is largely avoided (28). Provided the baseline level of lung function is sufficient to permit a 20% drop in FEV_1 the technique is believed safe.

Attempts have been made to standardise bronchial challenge procedures (27, 29, 30). However protocols in use currently vary in terms of subject eligibility for challenge, provocation agent, mode of delivery, starting and maximum concentrations and expression of response (31). Although specific conductance was used in some of the early studies (32, 33), FEV_1 is now agreed as the standard measure of lung function because of its greater reproducibility (30). Response can be expressed in terms of the dose delivered (if the output by weight of the nebuliser, drug concentration and number of breaths are known), or just drug concentration. In the past epidemiological studies have tended to express their results in terms of dose delivered (PD_{20}) (34, 35) whereas

protocols in clinical practice usually use concentrations (PC_{20}) (26). Results can also be expressed in terms of the cumulative dose or concentration delivered, or non-cumulatively as the lowest dose or concentration causing the drop in FEV_1. Whether this is important is debatable as it has been suggested that methacholine has only a small cumulative effect and that the effect of histamine is non-cumulative (36).

1.2.4 Clinical significance

Airway hyperresponsiveness has a unimodal distribution in the general population (37) and therefore its measured prevalence depends on the method and cut off point used. Early studies reported a good correlation between asthma symptoms and BHR (26, 38). Using a cut off level for PC_{20} (histamine) of 8 mg/ml Cockcfoft *et al* correctly identified all symptomatic asthmatics in the group studied, but 50% of asymptomatic subjects also had a PC_{20} below 8 mg/ml (39). This result may have occurred because the cut off point was set too high or the symptoms were intermittent or not recognised/reported by the subjects. Other studies have shown that this group are more likely to be atopic and demonstrate peak flow variability (40) or to have a family history of asthma. Despite these issues, in patients with appropriate asthmatic symptoms and BHR, a significant correlation is reported to exist between BHR and asthma severity (26), and with the amount of treatment needed to control symptoms (41). In clinical and research practice a PC_{20} (histamine or methacholine) of $\leq$ 8mg/ml, associated with an appropriate clinical history, is most commonly used in support of a diagnosis of asthma (30).

The dose response curve to a bronchoconstrictor agent from an asthmatic differs from that of a normal subject in three ways (25). The curve is shifted to the left, i.e. initiation of constriction occurs at lower concentrations (termed hypersensitivity). The slope of the curve is steeper (termed hyperreactivity) and there is a greater maximal response. See Figure 1-1. Healthy subjects may not constrict by 20% even at very high doses, suggestive of a plateau that is not seen in asthmatics (42, 43).

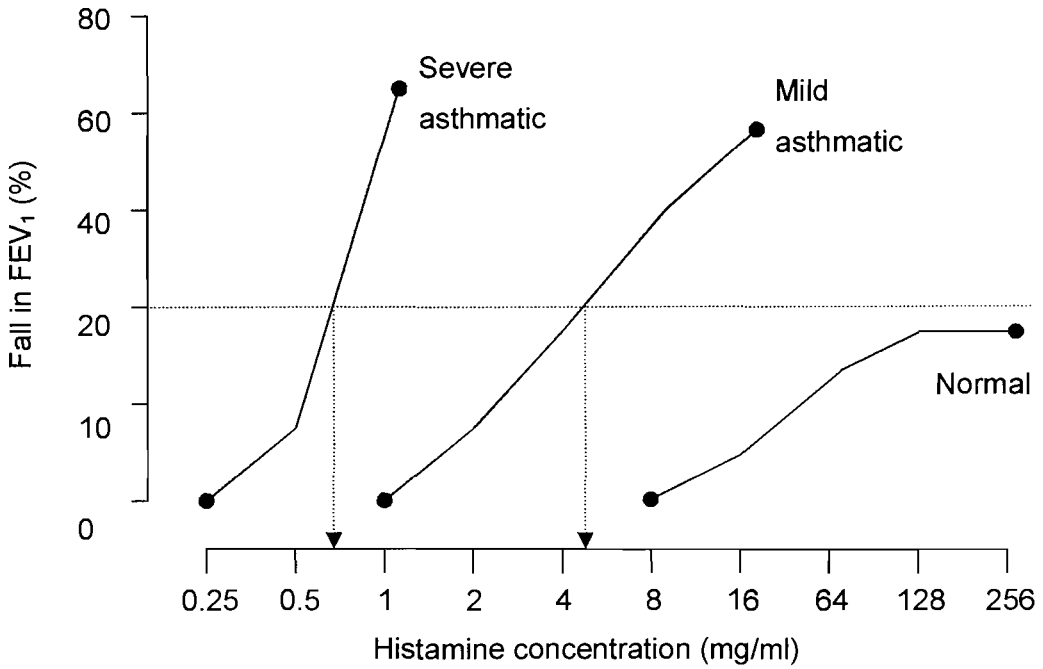


Figure 1-1. Dose response curves in bronchial hyperresponsiveness

Typical dose response curves in the assessment of bronchial responsiveness (PC_{20}). The change in FEV_1 *vs.* baseline for each concentration of histamine is calculated. Asthmatics show a reduced threshold response, have an increased sensitivity (slope) and greater maximal response to the bronchoconstrictor.

1.2.5 Genetic associations

It has been recognised for some time that familial clustering of cases occurs not just in asthma but also with airway hyperresponsiveness (44, 45). This could reflect a shared genetic predisposition, environmental factors or perhaps most likely both. A study to examine the genetic basis of BHR in asthma by the comparison of mono and dizygous twins suggested that whilst there may be a genetic basis for its development, environmental factors were of greater importance (46). Another twin study suggested that asthma has a heritability of up to 79% (47). Linkage studies have identified genes associated with BHR on chromosome 5q (near a locus that regulates serum IgE levels) (48), chromosome 11q (near a locus encoding the β subunit of the high affinity IgE receptor) (49) and chromosome 20p (gene encoding ADAM33 protein) (50). In combination these studies suggest a number of genes in combination with the environment influence the asthmatic phenotype.

1.3 Airway remodelling

1.3.1 Overview

The concept of reversible airway obstruction in response to a variety of endogenous and exogenous stimuli led to the belief in the 1970s that asthma was primarily a disorder of bronchial smooth muscle. Treatments at that time were directed towards bronchial smooth muscle relaxants such as β_2 agonists and xanthenes. Over the following 10 - 15 years increasing evidence became available from *in vivo* studies using broncho alveolar lavage (BAL) and endobronchial biopsies that particular inflammatory changes occur in the airways of asthmatics, involving eosinophils, mast cells and activated T cells .(51-56). Many long term studies have demonstrated the superiority of anti inflammatory treatment with corticosteroids, over bronchodilators alone, in improving symptoms, BHR, FEV_1 and PEF variability in adults and children (57-61). Subsequent clinical guidelines have recommended corticosteroids as a key component in asthma management (3, 62). We now appreciate a third level of complexity, since the discovery that a number of important structural changes occur in the airway wall, collectively termed airway remodelling (63). These changes may account for the persistence of lung function abnormalities despite clinical remission (64) and effective control of eosinophilic airway inflammation (58, 65). Duration of asthma prior to the introduction of inhaled steroids has been shown to be inversely related to improvement in lung function after their introduction (66). Delaying anti-inflammatory treatment reduces the chances of returning lung function to normal (60). In chronic disease the increase in BHR is often only partially or non-responsive to treatment with corticosteroids (57, 67). This evidence suggests asthma can result in irreversible airflow obstruction.

In normal tissues acute inflammation in response to injury of any kind leads to the beneficial processes of repair and restoration of normal structure and function. In asthma however, although the degree of inflammatory activity may vary with time it is never completely suppressed. This chronic inflammation and associated repair processes occur in parallel resulting in airway remodelling (63). These structural changes within the airways encompass desquamation (68), goblet cell proliferation (69), collagen deposition in the reticular layer of the sub epithelial layer (70), hyperplasia of mucous glands (71), hypertrophy and hyperplasia of smooth muscle and hyperplasia of bronchial blood vessels (4, 72-74). See Figure 1-2.

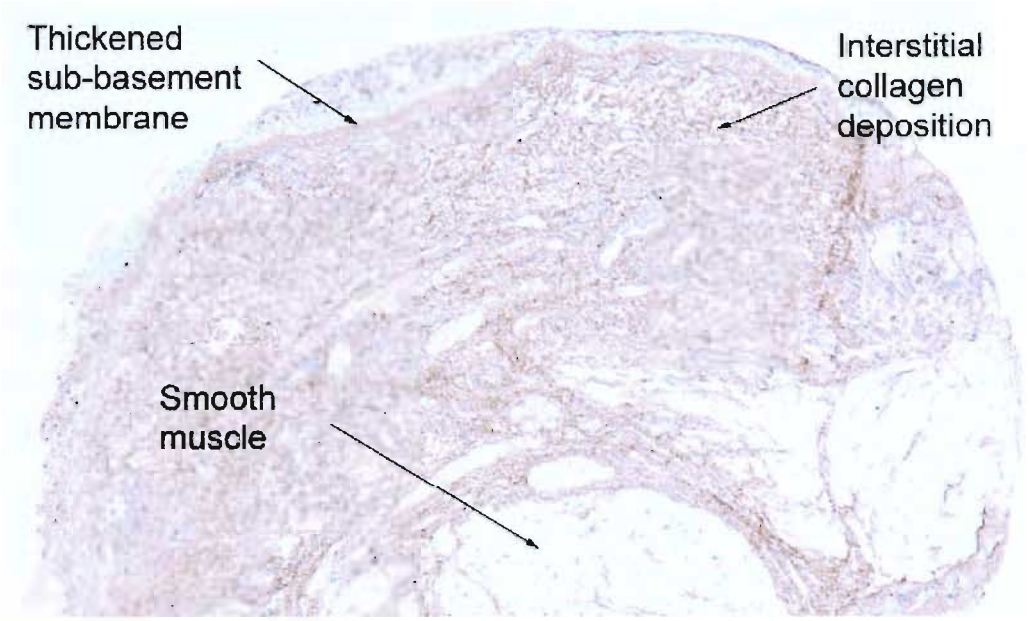


Figure 1-2 Example bronchial biopsy from an asthmatic subject

The biopsy is stained to highlight collagen I, and demonstrates some of the airway changes associated with airway remodelling.

Early studies demonstrated remodelling in large and small airways in lung tissue obtained at post mortem from asthmatics dying as a result of their asthma or of unrelated causes (75-77). Bronchial biopsies from the central airways have confirmed these changes *in vivo* (78) and more recently transbronchial biopsies, that have by chance included small airways, have also shown evidence of remodelling in the periphery of the lung (79). Biopsies from asymptomatic subjects with increased BHR have also shown a degree of remodelling between normal and asthmatic subjects (80). Although bronchial biopsies are able to demonstrate several qualitative changes in the bronchial wall they are of insufficient depth to assess precise changes in the airway smooth muscle distribution or the total wall thickening.

1.3.2 Airway thickening

The observation that total airway thickness is increased in asthmatics was first noted by Huber and Koessler in their post-mortem study of 6 subjects with fatal asthma and 7 non asthmatics, published in 1922 (76). This and other reports from the 1950s and 1960s (81-83) relied on qualitative descriptions or imprecise quantification of the changes seen. The later recognition that the internal perimeter of an airway remains relatively constant at different lung volumes and degrees of smooth muscle shortening permitted greater confidence in the reporting of airway thickness in explanted tissue

(84). Using this method an increase in total airway wall area was confirmed in cartilaginous and membranous airways of all sizes (85).

Airway wall thickening has also been documented at post mortem in subjects dying with asthma but from other causes (75, 77). Carroll *et al* found the thickening in fatal asthma to be most marked in the central airways, though it was present throughout. In non-fatal asthma the changes were mainly confined to the smaller peripheral airways (<2mm in diameter). The increase in thickness has also been shown to be less marked than in fatal asthma (77) and in younger subjects (86).

1.3.3 Epithelial damage

At post mortem in fatal asthma the airways are filled with mucus and sloughed epithelial cells. The usual pseudostratified epithelium is denuded leaving exposed basal cells (68). Whilst these changes could be put down to the preservation techniques used in a post-mortem study, there is also evidence from *in vivo* work. An increase in columnar cells in BAL from asthmatics has been reported (87), though others have found no difference (88). The degree of epithelial shedding has been correlated with BHR in some (71, 89) but not all studies (53, 90).

1.3.4 Reticular basement membrane thickening

A characteristic finding in asthmatic airways is the deposition of excess connective tissue in the reticular basement membrane (RbM) underlying the epithelial layer. This was noted in the early post mortem studies (68, 91), and has been subsequently confirmed using *in vivo* biopsy techniques via rigid (92) and fibre optic bronchoscopes (70, 89). The changes are present early in the course of the disease (93, 94) and occur to some extent in subjects with rhinitis (95) or BHR alone (80) without symptoms of asthma. Electron microscopy has demonstrated that the lamina rara and lamina densa, comprising the true basement membrane onto which the basal layer of epithelial cells are attached, is of normal thickness (96). Deposition of collagens I and III, and fibronectin occurs in the lamina reticularis, which lies immediately beneath the true basement, increasing its depth by 1.5 – 2 fold over the normal thickness (70, 97). An increase in the glycoprotein tenascin has also been reported (98) and another glycoprotein, laminin, has also been shown to be present at the superficial margin of the basement membrane (99).

RbM thickeness can be easily measured in airway biopsies using electron microscopy, though a simple staining technique such as toluidine blue and light microscopy has been shown to be less expensive and just as accurate (100). Immunohistochemistry using antibodies to RbM components such as collagen I has also been used (101). Proliferating myofibroblasts are thought to be responsible for new collagen production and their numbers correlate with the thickness of the RbM (96). RbM thickening is frequently used as an index of airway remodelling and its presence is accepted as a universal feature in asthma, the question of whether the increase in thickness correlates with asthma severity remains disputed. as some workers have found this to be the case (97) whilst others have not (86, 101).

1.3.5 Smooth muscle hypertrophy and hyperplasia

An increase in airway smooth muscle (ASM) mass has been reported to occur throughout the bronchial tree in cases of fatal and not fatal asthma (71, 73, 83, 102). Ebina *et al*, reporting a study of post mortem tissue, suggested that the increase in ASM occurs in one of two patterns. In some subjects, "type I", the increase is due to hyperplasia restricted to the larger central airways, whilst in others, "type 2", the hyperplasia in the central airways is mild but hypertrophy occurs throughout the bronchial tree especially in the smaller peripheral airways (74). In addition to these morphological changes some workers have described changes in the functional capacity of ASM in asthmatics (103). An increase in maximal shortening velocity has been reported after incubation of ASM from controls with serum from atopic asthmatics (104). This could explain why asthmatics appear to lose the normal transient bronchodilatation effect of deep inspiration (105).

1.3.6 Mucus gland metaplasia and goblet cell hyperplasia

In normal subjects mucous glands are confined to the central airways. In asthmatics these glands are larger and more numerous with their distribution extending more peripherally out into the bronchioles. These changes were observed in the studies of post mortem material (75, 83). A bronchoscopic biopsy based study also found mucous gland hyperplasia in asthmatics compared to controls, though no correlation was found with disease severity (71).

1.3.7 Vascular changes

Blood vessel dilatation was described as a striking feature in many cases of fatal asthma at post mortem (83). A subsequent quantitative study of post mortem and surgically resected tissue from asthmatics and controls confirmed an increase in the proportion of the submucosa occupied by vessels in asthmatics (77). Another post mortem study reported more larger and fewer smaller submucosal vessels in the central airways of cases of fatal asthma over non fatal asthma and controls, though the total cross sectional vascular area did not differ between the 3 groups (106). Studies involving bronchoscopic biopsy techniques have confirmed an increase in both the size and number of vessels in specimens from asthmatics over controls in mild (107, 108) and more severe disease (109). The extent to which vascular enlargement contributes to total airway thickness remains unclear.

1.3.8 Structural collagens and elastin

Interstitial collagen (primarily types I, III and V) deposition is not confined to the RbM. It is also increased throughout the submucosal area (100). The close proximity of such rigid material to ASM bundles may have greater functional implications than the increase in RbM thickness alone (5).

Elastin fibres within airway walls have an important role physiologically as they permit elastic recoil to drive expiration. There is disagreement as to whether the distribution and configuration of elastin fibres differs between asthmatic and controls. Godfrey *et al* found no difference in the elastin fibre content between cases of mild, severe and fatal asthma when compared with controls (110). Bousquet *et al* however found fragmentation of the subepithelial elastic fibres and in the deeper areas the fibres were often patchy, tangled and thickened (111). Looking at post mortem specimens from cases of fatal and non fatal asthma, and healthy controls Carroll *et al* found that in fatal asthma the area of elastin fibre bundles was increased but that their elastin content was reduced (112).

1.3.9 Proteoglycans

Although difficult to quantify experimentally there is evidence to support increased submucosal deposition of extracellular matrix glycoproteins including lumical, biglycan, versican, decorin and fibronectin in asthmatic airways, with increases in the first three of these being shown to correlate with BHR (113, 114). These not only contribute to

the wall thickening directly but are believed to have an important active role in cell – cell signalling and act as a reservoir for cytokines and growth factors (115).

1.4 Orchestration of airway remodelling

Remodelling of the airways in asthma involves structural changes in the epithelium, (myo)fibroblasts, smooth muscle, extracellular matrix and reticular basement membrane. The process is mainly orchestrated by a complex interaction of inflammatory and structural tissue cells through their release of a variety of mediators, cytokines and chemokines. Epithelial changes in particular are believed to play a vital role in remodelling. A genetically sensitive epithelium, which is then damaged by toxic mediators, is thought to be responsible for driving the subsequent thickening of the reticular basement membrane and submucosal alterations of collagen, elastin and smooth muscle fibres. This interaction is known as the epithelial-mesenchymal trophic unit (116).

Goblet cell numbers, mucin production and the predominant mucin gene MUC5AC expressed are increased in asthmatics (117). The finding of increased epidermal growth factor receptor (EGFR) expression in asthma supports a proposed central role of EGF in the epithelial changes in asthma (118). The 3v isoform of CD44 has been found to be increased in damaged epithelium and binds to HB-EGF more efficiently thereby possibly exaggerating the repair response (118). EGFR immunoreactivity correlates with reticular basement membrane thickening in asthmatics despite treatment with corticosteroids (119). The cytokines IL-4 and IL-13 and the house dust mite allergen Der p 1 have been shown to enhance TGF-β release from atopic asthmatic primary bronchial epithelial cells in culture (120). IL-13 knockout mice show a reduction in airway inflammation, mucous cell hyperplasia and subepithelial fibrosis, with a similar but less marked effect in IL-4 knockouts (121).

TGF-α has been shown to induce mucin production via EGFR activation (122) and TGF-β2 to cause activation of myofibroblasts to secrete matrix proteins important in remodelling (123). This evidence provides a potential link between allergen exposure of the epithelium, Th2 cytokines and airway remodelling in asthma.

1.5 Mathematical modelling of airways

Mathematical models suggest airway thickening will enhance BHR. It was initially proposed by Moreno *et al* that small variation in airway thickness, that have little effect on baseline airway resistance, could enhance maximal narrowing to contractile agonists (124) See Figure 1-3. A study by James *et al* of wall thickness data obtained from morphometric analysis of airways obtained at post mortem from 18 asthmatics and 23 controls found that the wall area was greater in the membranous and cartilaginous airways of asthmatic patients and, according to their model, the asthmatics would require 10 – 15 % less ASM shortening to cause airway closure in membranous and cartilaginous airways respectively (72).

The mathematical modelling process was further developed by Wiggs *et al*. Based on proposals by Weibel (125) they modelled the lungs as a symmetrical dichotomously branching tree with 23 generations, though only generations 0 - 16 were used in the final analysis as these represent the conducting airways that affect resistance to airflow *in vivo*. Given data for AWT, airway diameter, ASM shortening and the length of each airway generation the model gave a predicted estimate of total and regional airway resistance (126). Using post-mortem morphometric data from the earlier study on airway closure the same authors applied the model to the study of BHR in asthma and COPD. The model was adjusted to accept figures for AWT, the proportion of the airway circumference occupied by ASM, the proportion of airway tissue internal to the ASM layer and the percentage of ASM shortening (PMS_{max}). Values for PMS_{max} could not be obtained from morphometry and values of 20-40% were used. The authors comment on the difficulty of obtaining accurate *in vivo* data for PMS_{max} and the only study quoted is by Okazawa *et al* who attached piezoelectric transducers to the posterior tracheal wall in dogs and measured smooth muscle shortening in response to vagal stimulation where the mean shortening observed was 28% (127). Loss of elastic recoil was simulated by deflating the lung along its pressure-area curve, which has the effect of reducing tension on airway parenchymal supports. The study concluded that loss of elastic recoil and an increase in AWT could at least partially explain BHR in asthma. In a further modification of their model the same group investigated the relative effects of submucosal, ASM and adventitial thickening. Whilst all enhanced narrowing they concluded that increases in ASM mass would have the greatest effect on BHR (128).

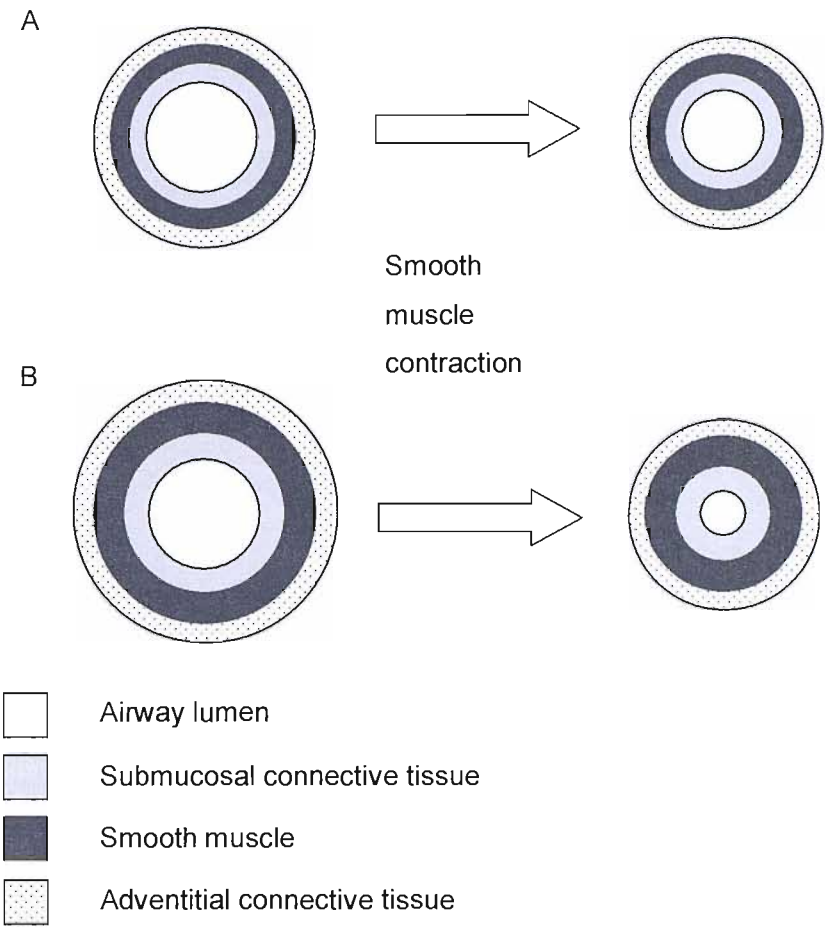


Figure 1-3 Smooth muscle contraction and luminal area

Schematic diagram showing of the effect of smooth muscle contraction on luminal area in normal (A) and asthmatic airways (B). Resting luminal area is similar in both but smooth muscle contraction amplifies the luminal narrowing in asthmatics. In B the thickened submucosa internal to the smooth muscle is non-compressible and this impinges on the lumen whilst thickening of the adventitia reduces the opposing radial forces provided by the lung parenchymal supports which served to keep the airway open.

In vitro studies of airways treated with bronchoconstrictors demonstrate that the lumen does not remain smooth and circular as shown in Figure 1-3. Rather the mucosa buckles and forms folds that penetrate into the airway lumen. This folding pattern may influence the amount of luminal obstruction associated with smooth muscle activation. Using a two-layer composite model of the airway Wiggs *et al* investigated the factors that determine the mucosal folding pattern and how this could be altered as a result of changes in the thickness or stiffness of the different layers that comprise the airway wall. They found that the most critical physical characteristic to be the thickness of the thin inner layer of the model. Thickening of this inner layer in the model could

represent the *in vivo* deposition of subepithelial collagen seen in asthma. See Figure 1-4 (129).

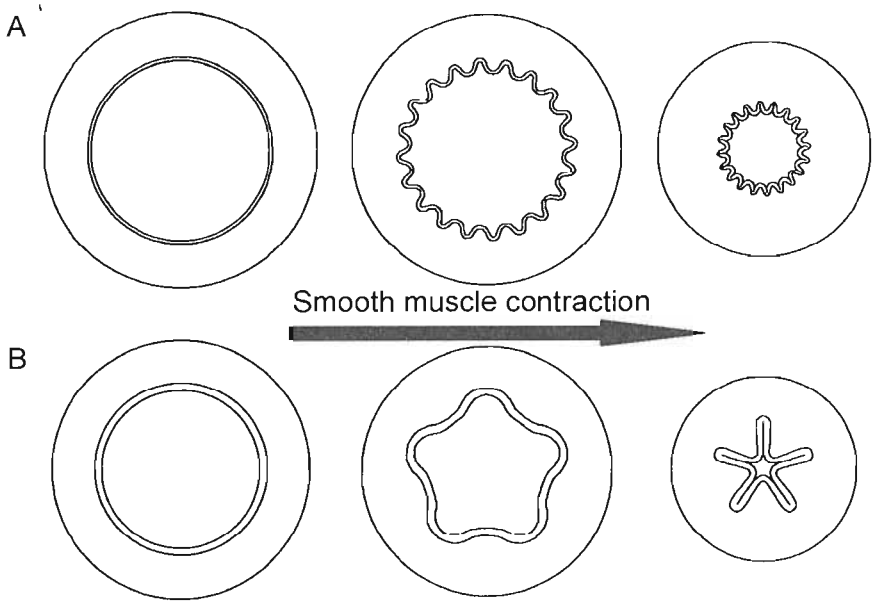


Figure 1-4 Effect of thickening/stiffening of the submucosa on mucosal folding
"A" represents the normal thickness and "B" the thickened reticular basement membrane seen in remodelled asthmatic airways. Thickening of this layer reduces the number of folds and enhanced luminal narrowing. Adapted from reference (129).

1.6 Techniques for assessment of airway wall thickness

In order to assess total AWT both the luminal and adventitial boundaries must be identified and unfortunately standard bronchial biopsies are of insufficient depth. The ideal method would be highly accurate yet non-invasive and requiring no exposure to radiation. With current technology this remains illusive. A number of possible methods have been used previously and these are described below.

1.6.1 Post mortem studies

As described above the initial observational studies by Huber and Koessler in 1922 showed that total airway thickness was increased in fatal asthma (76). Subsequent studies employing more quantitative techniques found similar findings in fatal and non fatal asthma (72, 75, 77, 86). There are a number of problems with these necropsy studies. All looked at relatively small numbers of asthmatics (maximum of 24 subjects) and little clinical data on spirometry or BHR is provided. Many subjects were selected because they had died of their disease and this in itself makes them atypical. Clearly post-mortem material is obtained after death and the changes observed will include

those occurring around and after death. The process of tissue fixation is known to cause differential tissue shrinkage (130) which may cause distortion of the airway anatomy. Post mortem work can also never be applicable to intervention studies into airway remodelling. For these reasons other researchers have investigated alternative *in vivo* methods to study of airway thickness.

1.6.2 Surgically resected material

It would be unethical to operate on asthmatic subjects purely for research purposes, however a few cases have occurred where a peripheral lung cancer has been removed from a patient know to have asthma and this tissue has become available. The study by Kuwano *et al* though primarily studying post mortem tissue, included 4 out of 15 cases where material came from patients undergoing this type of surgery, but the results are combined. Other studies have concentrated on using this technique to demonstrate inflammation in the peripheral airways of asthmatics rather than remodelling (131, 132). In all these studies the numbers of subjects enrolled are necessarily small and most subjects are older and have a history of smoking.

1.6.3 High resolution computed tomography

The advent of High Resolution Computed tomography (HRCT) provides an x ray based technique that allows a high degree of anatomical detail to be seen and can be used to assess total AWT. Early studies employing the technique looked at the general appearances of the lungs in asthmatics verses healthy controls with findings such as a greater frequency of bronchial dilatation (133) (This study included a high proportion of smokers) and more frequent bronchiectasis, emphysema and linear shadowing in asthmatics than controls (134).

Subsequent studies looked at AWT in normal controls and asthmatics with various assessments of disease severity and airway inflammation. Spirometry, PEF variability, histamine/methacholine hyper responsiveness and bronchial biopsy data have all been compared to bronchial wall thickness. The results are not entirely conclusive and highlight some of the limitations of the technique. Boulet *et al* failed to show evidence of airway wall thickening in all asthmatics (135). More recent studies have demonstrated airway wall thickening in asthmatics compared to controls, especially in the smaller airways (136-140). Whilst HRCT allows total wall thickness to be

measured it is unable to identify different components and layers within the airway wall (140).

The protocols used in these HRCT assessments vary in two main ways. Firstly either the investigators studied one predetermined bronchus (135, 139, 141) or they assessed all airways, seen in the correct orientation, within 5 pre selected slices through the lung and related to clear anatomical landmarks (136, 138, 140, 142). Secondly they differ in their calculated measures of AWT. One study related wall area (WA) to body surface area (BSA) (139) but most relate wall thickness to the external airway diameter, airway area or both. Wall area can be obtained either directly by subtraction of the luminal area from the total (external) airway area using image analysis software or calculated using mathematical formulae from measured airway diameters or idealised airway diameters derived by tracing the internal and external perimeters. See Table 1-1 for summary of HRCT studies of AWT in asthma.

Raw data from the CT scanner is measured in Hounsfield units (HU) representing the density of an individual block of tissue in space or voxel. Each voxel has x and y dimensions represented on the 2D image but also holds density data on the z dimension or slice thickness. Values of -1000 HU to +1000 HU are returned, with 0 HU representing the density of water. The eye cannot discriminate between 2000 shades of grey so images are viewed over a 16 shade greyscale based on a "Level" (the value in HU of the middle shade of grey) and "Window Width" (the range of HU over which the 16 shades of grey are spread) setting. Visualisation of the lung parenchyma requires a window level in the negative HU range since aerated lung tissue is less dense than water.

Window width and Level

There is a consensus that window width and level settings are critical to the identification of wall/luminal boundaries (143). Studies both in excised canine lungs (144) and with phantoms (e.g. plastic tubes in styrofoam or sweet potato) have helped to determine the optimal settings (143) and a window level of -450 HU has been used by most investigators. The window width is less important but a figure of 1500 HU has

Paper	Physiological assessments	HRCT measures	Findings
Boulet *et al* 1995 11 asthmatics (variable air flow limitation) 13 asthmatics (fixed air flow limitation) 10 controls	FEV_1 Bronchodilator response PEF record PC_{20} (Methacholine)	Wall thickness/diameter ratio (T/D)	T/D ratio correlated with PC_{20} in fixed airflow obstruction group only.
Okazawa *et al* 1996 6 asthmatics (mild) 6 controls	FEV_1 PC_{20} (Methacholine)	Direct wall area (WA) and %WA (Direct measures of area by image analysis software)	%WA greater in asthmatic group in smaller airways (< 6mm Φ)
Park *et al* 1997 57 asthmatics 19 COPD, 10 controls	FEV_1 PC_{20} (Methacholine)	T/D ratio	No correlation found between T/D and pulmonary function or PC_{20}
Awadh *et al* 1998 40 asthmatics (mild – severe) 14 controls	FEV_1 Bronchodilator response Lung volumes (body box) Skin prick tests	T/D ratio Idealised %WA (Derived from short axis diameter measurements)	T/D and %WA were significantly greater in the moderate/severe than the controls and mild asthmatics groups
Niimi *et al* 2000 81 asthmatics (mild – severe) 28 controls	FEV_1 Total serum IgE Serum ECP Skin prick tests	Direct WA, WA/BSA and %WA (Direct measures of area by image analysis software)	WA, WA/BSA and %WA greater in all except mild intermittent asthmatics than controls Good inter and intra observer variability of scan assessment
Little *et al* 2002 49 asthmatics (moderate - severe)	FEV_1 TLCO ATS asthma severity score PC_{20} (Histamine) Exhaled NO Induced sputum – cell counts	T/D ratio Idealised %WA derived mathematically from internal and external short axis diameters	%WA and T/D correlated with asthma severity score %WA and T/D inversely correlated with TLCO Good intra, poor inter observer variability of scan assessment
Kasahara *et al* 2002 49 asthmatics (Given oral steroids for 2 weeks to reduce inflammation) 18 controls	FEV_1 Bronchodilator response Bronchial biopsies - Basement membrane (Rbm) thickness	Direct %WA and idealised %wall thickness (%WT) (%WT from diameters derived from measured perimeters)	%WA and %WT higher in asthmatics %WA and %WT correlated with Rbm thickness and inversely with FEV_1
Niimi *et al* 2003 23 asthmatics inhaled steroid + 22 asthmatics inhaled steroid -	Airway sensitivity and reactivity (Methacholine) Induced sputum – eosinophils	Direct WA, %WA, T/BSA and WA/BSA (Automated analysis)	Reactivity negatively correlated with WA/BSA in both groups. Sensitivity not correlated with any measures of airway thickness

Table 1-1 Summary of previous work utilising HRCT to measure AWT

been generally used as it allows the identification of major lung structures for orientation purposes (145).

Inter and Intra observer variability

Interpretation of the airway boundary is observer dependent, which has lead to problems with inter observer variability. Two studies have looked at this problem by repeated measurements of bronchi by different observers and by the same observer after a time interval (139, 142). The researchers compared their results according to the methods described by Bland and Altman (146). Both studies found no significant difference in measures by the same observer but one found a significant degree of inter observer variability (142) since different observers may interpret the edge of the airway wall to lie in different positions. Two recent studies have overcome this problem by used a computer based automated technique to assess wall area of a single large airway (141, 147).

Bronchus orientation

HRCT data is collected in an axial manner and airways are not always cut at 90°. This causes the lumen to look non-circular and reduces its apparent luminal size by an effect known as volume averaging (see Figure 1-5). A number of authors have tried to overcome this by 3D reconstruction of multiple slices to generate an apparently perpendicular cut through across the bronchus (148, 149) at some cost in terms of image clarity. The advent of multi-slice scanners will allow more data to be collected during a single breath hold, theoretically allowing improvements in reconstructed images.

Volume Averaging

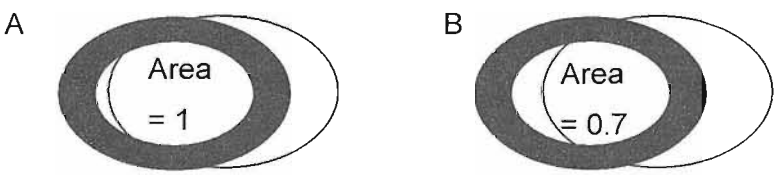


Figure 1-5 The effect of volume averaging in CT imaging

The cross sectional appearance of an airway lumen in an HRCT slice may be distorted if the airway is cut obliquely or the slice thickness is increased. This can be seen by comparing the apparent luminal area in A with the more obliquely cut lumen in B

Radiation dose

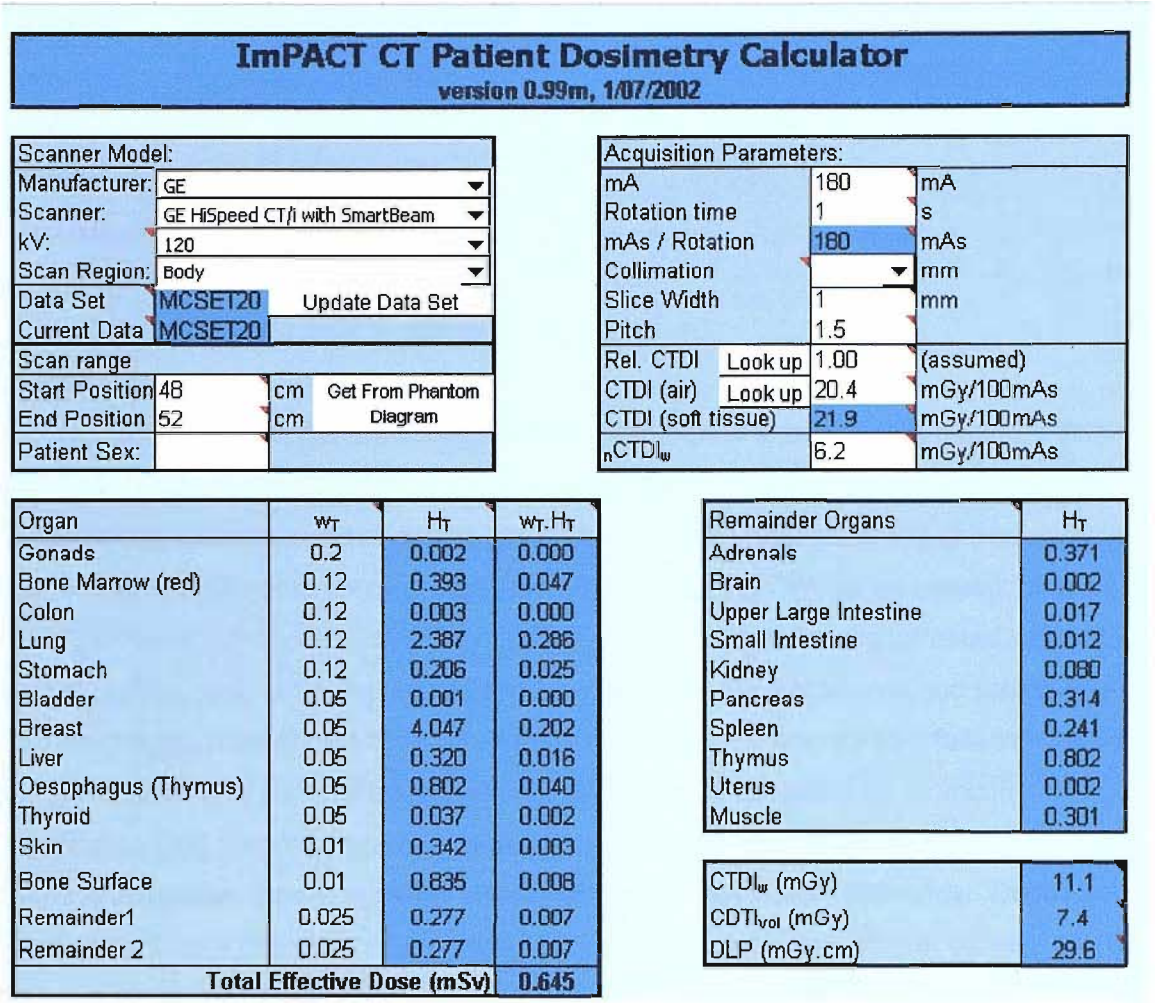
The radiation dose associated with a CT scan of the thorax in clinical practice is not insignificant with a full series of thick and high resolution sections on a modern scanner exposing the individual to approximately 8-10 mSv. Examples of the radiation exposure from a number of common radiological procedures are shown in Table 1-2.

Diagnostic Procedure	Typical effective dose (mSv)	Equivalent No of chest xrays	Approximate equivalent period of natural background radiation*
Chest (PA film)	0.02	1	3 days
Thoracic spine	0.7	3.5	4 months
Barium enema	7	350	3.2 years
CT Chest	10	400	3.6 years
CT abdomen or pelvis	10	500	4.5 years

Table 1-2 Typical radiation doses from diagnostic medical procedures

* UK average background radiation = 2.2 mSv per year
(Regional averages range from 1.5 to 7.5mSv per year (150))

I quoted a maximum radiation dose of 2mSV to the ethics committee, on the basis that only a limited area of lung tissue would be scanned, but calculations have shown the exposure in this study to be much less than this, at 0.645mSv. The dose calculations were made using the ImPACT patient CT dosimetry calculator (151). The reference Monte Carlo dose data sets (report SR250), required by the ImPACT program, were obtained from the National Radiological Protection Board (152). By providing data about the type of CT scanner and acquisition parameters organ specific and a total effective radiation dose may be estimated. See Figure 1-6.



ImPACT CT Patient Dosimetry Calculator
version 0.99m, 1/07/2002

Scanner Model:			
Manufacturer:	GE		
Scanner:	GE HiSpeed CT/i with SmartBeam		
kV:	120		
Scan Region:	Body		
Data Set	MCSET20	Update Data Set	
Current Data	MCSET20		
Scan range			
Start Position	48	cm	Get From Phantom
End Position	52	cm	Diagram
Patient Sex:			

Acquisition Parameters:			
mA	180		mA
Rotation time	1		s
mAs / Rotation	180		mAs
Collimation			mm
Slice Width	1		mm
Pitch	1.5		
Rel. CTDI	Look up	1.00	(assumed)
CTDI (air)	Look up	20.4	mGy/100mAs
CTDI (soft tissue)		21.9	mGy/100mAs
$_nCTDI_w$		6.2	mGy/100mAs

Organ	w_T	H_T	$w_T.H_T$
Gonads	0.2	0.002	0.000
Bone Marrow (red)	0.12	0.393	0.047
Colon	0.12	0.003	0.000
Lung	0.12	2.387	0.286
Stomach	0.12	0.206	0.025
Bladder	0.05	0.001	0.000
Breast	0.05	4.047	0.202
Liver	0.05	0.320	0.016
Oesophagus (Thymus)	0.05	0.802	0.040
Thyroid	0.05	0.037	0.002
Skin	0.01	0.342	0.003
Bone Surface	0.01	0.835	0.008
Remainder1	0.025	0.277	0.007
Remainder 2	0.025	0.277	0.007
Total Effective Dose (mSv)			**0.645**

Remainder Organs	H_T
Adrenals	0.371
Brain	0.002
Upper Large Intestine	0.017
Small Intestine	0.012
Kidney	0.080
Pancreas	0.314
Spleen	0.241
Thymus	0.802
Uterus	0.002
Muscle	0.301

$CTDI_w$ (mGy)	11.1
$CDTI_{vol}$ (mGy)	7.4
DLP (mGy.cm)	29.6

Figure 1-6 ImPACT dose calculations for limited HRCT scans

Screenshot from ref (151), showing total effective radiation dose received by subjects

1.7 Medical Ultrasound

1.7.1 Ultrasound physics

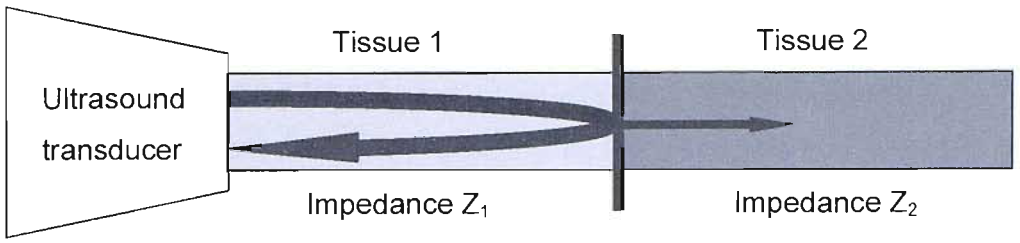
The medical imaging application of ultrasound developed in the 1950s from the World War II advances in SONAR (sound navigation and ranging). Unlike X rays, which are a form of electromagnetic radiation, ultrasound uses high frequency sound waves, by definition above the range audible to the human ear (> 20,000 Hz). Ultrasound pulses are mechanical and therefore require a medium for transmission with propagation occurring by displacement of molecules within it. The pulses travel relatively slowly through tissues, with a mean speed of 1540 m/s (compared with 3 x 10^8 m/s for X rays), allowing electronic circuitry to detect minor delays in signal propagation. See Table 1-3 for the velocity of sound through various tissues.

Tissue	Velocity (m/s)
Air	330
Fat	1450
Water	1480
Skull bone	4080

Table 1-3 Velocity of sound in different tissues

Ultrasound pulses are generated by electrical vibration of a crystal (the piezo-electric principle), typically lead zirconate-titanate, which can also act as a receiver. After each pulse the transducer reverts back to "listen" for returning echoes reflected back before converting them back to an electrical signal for processing. When an ultrasound wave meets an interface between tissues of different density, the wave is reflected, refracted and absorbed. Only reflected sound can be sensed by the transducer and processed into an image. Refraction alters the path of the ultrasound energy so it fails to return to the transducer and some energy is absorbed due to friction between molecules within the propagating medium, resulting in its conversion into heat. Attenuation refers to all these propagation losses together and it increases linearly with frequency. Depth or axial resolution is the ability of the beam to separate two objects lying in tandem along the axis of the beam. It depends of the wavelength (and hence frequency as wavelength = 1/*frequency*) which must be less than twice the separation between the objects for them to be resolved independently. Whilst resolution is therefore dependent on frequency, penetration is dependent on attenuation, which is inversely related to the ultrasound frequency.

Ultrasound waves directed at right angles to a smooth interface larger than the width of the beam will be partially reflected back toward the source. The strength of the reflected sound wave, or echogenicity, will depend on the difference in "acoustic impedance" between the materials either side of the interface. The acoustic impedance of a tissue is related to its density and the velocity of the sound wave passing through it. The greater the mismatch in acoustic impedance between two adjacent tissues the more reflective will be their boundary (see Figure 1-7). Very reflective interfaces such as bone/tissue or air/tissue prevent imaging of weaker echoes from deeper, soft tissue interfaces.



$$\text{Reflection} = \left[\frac{Z_1 - Z_2}{Z_1 + Z_2}\right]^2$$

Impedance = density X velocity

Figure 1-7 Reflection at interface between tissues of different acoustic impedance

Handheld ultrasound devices in medical applications use a thick water based gel between the transducer and the skin surface to permit the ultrasound energy to pass freely into tissues and return. This is termed sonic coupling. In the lung a radial probe requires a fluid filled balloon sheath to be inflated between the transducer and the airway wall to improve sonic coupling and facilitate reliable imaging. The probe rotates through 360º within this balloon "window" to form an image of the airway and its surrounding structures, in the same way as a radar screen on a ship.

1.7.2 Endobronchial ultrasound

The experimental evaluation of endoscopic ultrasound with a radial probe was first described in 1989 by Silverstein and colleagues. They studied canine gut wall *in vitro* and *in vivo* and attempted to correlate the ultrasound and histological appearances (153). The basic components of a rotating ultrasound micro transducer at the end of an endoscopic probe producing 360° radial images are still the same today. Upper gastrointestinal ultrasonography has progressed rapidly and in many centres is the modality of choice in staging oesophageal cancer as mediastinal lymph node and vascular invasion can clearly be seen (154). The larger diameter of the gastroscope facilitates a separate biopsy channel alongside the light guide, camera and ultrasound channels making it possible to undertake interventional procedures in the oesophagus, stomach, pancreas and biliary tree (155). Lymph nodes in the mediastinum can also be accessed for biopsy in this way by the trans-oesophageal route. Radial ultrasound

probes have even been placed into the circulation - intravascular ultrasound has facilitated the identification of dynamic changes in pulmonary artery musculature in pulmonary hypertension (156). A study using a 20 MHz ultrasound probe in the assessment of skin cancer invasion found it to be more accurate than clinical palpation but it was not able to help identify the histopathological type (157).

The endobronchial application of ultrasound (EBUS) was first described in 1992 (158) and had been commercially available since 1999 (159). The radial endobronchial ultrasound probe is introduced via the biopsy channel of a standard bronchoscope. Linear array endobronchial ultrasound devices have also been built into the tip of the bronchscope allowing visualisation of the sector of tissue in contact with the transducer. A similar device has recently been marketed with a separate working channel allowing needle biopsy of structures in the lung and mediastinum under direct vision. At present EBUS is most widely used clinically in the assessment of surgical resectability in cases of non-small cell lung cancer. This modality of imaging is well suited to defining tumour extent within the bronchial wall and into neighbouring structures (160-162). A 20MHz probe has a penetration depth of 1.5-2 cm which has been determined to provide the optimum resolution with sufficient penetration to visualise the airway wall (153).

1.7.3 Measurement of airway wall thickness with ultrasound

EBUS is attractive as a technique with which to measure AWT *in vivo*. It does not involve radiation and provides the necessary resolution and penetration for imaging the airway wall. There are a number of potential problems that were identified before the commencement of the study:-

1. EBUS measurement of airway wall dimensions requires the introduction of a fibre optic bronchoscope into the airways. This is an invasive procedure and carries some risk. Bronchoscopy as a research tools has however been shown to be safe and allows biopsies to be taken at the same time to assess remodelling (163, 164).

2. When used *in vivo* a latex balloon is required around the transducer to provide sonic coupling with the airway wall. If free fluid is introduced into the airways it is quickly absorbed. Balloon inflation by the operator using a syringe may cause distortion of the airway wall and whilst this problem can be minimised by

stopping inflation as soon as the contact with the airway wall is achieved, it may be difficult to remove the effect completely. EBUS assessment of explanted tissue immersed in fluid would eliminate the necessity for the balloon sheath (160). A preliminary *in vitro* study of explanted material would be required to investigate the effect of the balloon sheath on airway wall structures.

3. Tissues identified with ultrasound do not have an ascribed density scale such as that developed by Hounsfield for CT. Window level and width must be adjusted by the operator to clarify the airway wall margins but only by experience with manual adjustments of these parameters would it become clear whether this was a problem experimentally.

4. Intra and inter observer variability of the technique is unknown.

1.8 Hypothesis

Thickening of the airway walls in asthmatic subjects over controls can be detected using endobronchial ultrasound and this correlates with an increase in bronchial hyperresponsiveness, as predicted by mathematical modelling, and markers of airway remodelling in endobronchial biopsies.

1.9 Aims of this study

1. To validate the technique of EBUS as a tool for the measurement of wall thickness in central cartilaginous airways.

2. To measure AWT by endobronchial ultrasound (EBUS) in mild to moderate asthmatics and to compare the findings to those from healthy non-asthmatic volunteers.

3. To compare measurement of AWT using endobronchial ultrasound with those obtained by HRCT scanning.

4. To test the hypothesis that AWT in asthma is an important determinant of bronchial hyper-responsiveness.

5. To assess the relationship between EBUS measures of AWT and measures of mucosal inflammation and airway structural change in endobronchial biopsies.

Chapter 2: Materials and Methods

2 Materials and Methods

2.1 Subject recruitment

Subjects were recruited from outpatient clinics at the Royal South Hants and Royal Bournemouth Hospitals and from a departmental database. The database contains names of asthmatics and healthy controls who have expressed an interest in being willing to participate in clinical trials, or have taken part in the past.

Approval for the study was obtained from the Southampton and West Hampshire local research ethics committee, the East Dorset local research ethics committee and the NHS Research and Development committees associated with Southampton University Hospitals NHS Trust and The Royal Bournemouth and Christchurch Hospitals NHS Trust.

Recruitment and initial characterisation primarily occurred in Southampton, except for 2 patients where it occurred in Bournemouth. Subjects were provided with an approved patient information sheet and gave written informed consent. General practitioners were informed of their patients' participation by letter.

2.1.1 Inclusion criteria

Age 18 – 65 years

Asthmatics

- A clinical diagnosis of asthma according to the GINA guidelines (3).
- Positive skin prick test to 1 or more common aeroallergens
- PC_{20} (Histamine) ≤ 8 mg/ml (non-cumulative)
- Stable symptoms not requiring a change in their medication in the 2 months prior to involvement in the study.

Controls

- No history suggestive of asthma or allergic disease.
- Normal spirometry
- Negative skin prick tests
- PC_{20} (Histamine) > 8 mg/ml (non-cumulative)

2.1.2 Exclusion criteria

Pregnancy or breast feeding

Significant co-morbidity (other than asthma in this group)

Smokers or ex-smokers with a history of > 10 pack years

Subjects requiring long acting β_2 agonists, leukotriene receptor antagonists or theophyllines.

2.2 Subject visits

Subjects attended for three scheduled study visits as shown in Figure 2-1 and the protocols for the assessments at each visit are described below.

Visit 1	**Visit 3**
Informed consent	Review of diary card (asthmatics)
Asthma/medical history	Spirometry
Spirometry	Salbutamol/Ipratropium nebuliser
Skin prick testing	Repeat spirometry (for reversibility)
Histamine challenge	Fibreoptic bronchoscopy
Diary card	EBUS assessment of airway wall thickness (RLL ± RML/LLL*)
	Endobronchial biopsies RLL
Visit 2	
Limited HRCT chest (RLL)	

Figure 2-1 Summary of study protocol subject visits.

RLL = posterior basal segment bronchus of right lower lobe. RML = lateral segment bronchus of middle lobe. LLL = posterior basal segment bronchus of left lower lobe. *A subgroup of control subjects had additional measurements taken from these extra bronchi

2.3 Spirometry

Spirometry was preformed using a dry wedge bellows spirometer (Vitalograph Ltd, Buckingham, UK), with the greatest FEV_1 and FVC achieved from three attempts being recorded. These values could come from different volume-time curves.

2.4 Assessment of bronchial responsiveness

Airway responsiveness was assessed using a modification of the method of Chai *et al* (27). Subjects were asked to abstain from using short acting β_2 agonists for at least eight hours prior to the histamine challenge. A baseline FEV_1 was obtained before five breaths from functional residual capacity to total lung capacity were made inhaling physiological saline then incremental doubling dilutions of histamine from 0.03 mg/ml – 8 mg/ml administered by an Inspiron nebuliser (Bard Ltd, Sunderland, UK) with an output of 0.33ml/min. FEV_1 measurements were made at one and three minutes after inhalation of each solution. A fall of >10% after the saline challenge precluded continuation of the histamine challenge. The challenge was stopped when a fall in FEV_1 of > 20% below the post saline baseline was achieved or the 8mg/ml concentration was reached. The non-cumulative concentration of histamine required to produce a 20% fall in FEV_1 (PC_{20}) was calculated by linear interpolation between the last two points of the log dose response curve administered.

2.5 Diary card recording

Asthmatic subjects were supplied with a peak flow meter (Miniwright, Clement Clarke International Limited, Harlow, UK) to measure their PEF at home for two weeks after entry into the study. The best of three blows in the morning and evening, before taking any bronchodilators, was recorded on a diary card with details of any chest symptoms or short acting bronchodilator therapy taken. The card was examined on visit 3 before proceeding with the bronchoscopy, to ensure acceptable asthma control (PEF diurnal variability < 10%, < 4 puffs β_2 agonists/day).

2.6 Skin-prick testing

All subjects underwent skin prick testing to assess atopic status. Six common aeroallergens (grass mix, cat, tree mix, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and aspergillus - Hollister-Stier Laboratories, Spokane, WA, USA). One drop of each solution was placed on the volar aspect of the forearm and pricked through with a sterile lancet (Entaco Ltd, Studley, UK). Appropriate positive and negative controls were included. The long axis wheal size was measured at 15 minutes. Positive atopic status was defined as a response of > 3 mm more than the control wheal, to one or more allergens.

2.7 HRCT imaging

CT scans were performed on a GE systems HiSpeed CT*i* helical scanner (GE Systems, Milwaukee, WI, USA) using a high resolution (bone) reconstruction algorithm, collimation thickness 1mm, pitch 1.5mm, 120 KV and 180mA. Images were captured with subjects in suspended full inspiration from the level of origin of the right middle lobe to approximately 2cm above the right hemi diaphragm with a field of view of 13cm coned down to cover the basal segments of the right lower lobe. This yielded 40 images over a 40mm vertical thickness of lung.

2.8 Fibreoptic bronchoscopy with EBUS

Bronchoscopic examination was performed at least seven days after histamine challenge. All subjects had given their written consent and the procedure was carried out according to the British Thoracic Society guidelines (165). Spirometry at baseline and 15 minutes after nebulised salbutamol (2.5mg) and Ipratropium bromide (0.5mg) was recorded prior to bronchoscopy in both asthmatic and control groups. Intravenous access was established and subjects were premedicated with *iv* atropine 0.6mg and *iv* fentanyl 50mcg to suppress coughing. Topical 10% and then 2% lignocaine spray (Astra pharmaceuticals, Kings Langley, UK) was applied to the pharynx and the bronchoscope (BFXT 40, Olympus, Keymed, Southend-on-Sea, UK) passed trans-orally with further 1% lignocaine instilled through the bronchoscope as necessary, up to a maximum of 5mg/Kg. Arterial oxygen saturation was monitored throughout (Ohmeda, Louisville, USA) and supplemental oxygen given by nasal cannulae if required.

The EBUS probe (PL2220-20, Hitachi medical systems, Japan), see Figure 2-2, was introduced via the bronchoscope working channel into the posterior basal segmental bronchus of the right lower lobe (this was repeated with the lateral segment of the middle lobe and the posterior basal segmental bronchus of the left lower lobe in the repeatability experiments). The balloon sheath was inflated with saline until 360° contact with the airway wall was just achieved and an image recorded. The balloon inflation/deflation/image capture process was performed three times at each site.

Three endobronchial biopsy specimens were taken from the right lower lobe subcarinae using 1.8mm disposable alligator cup forceps (BARD, Sunderland, UK order no. 100503) and immediately placed in ice cold acetone-containing protease

inhibitors (2mM phenylmethylsulphonyl fluoride with 20 mM iodoacetamide, Sigma) for subsequent processing into glycol methacrylate resin.

Post procedure, subjects were observed for at least 2 hours after the procedure and were not discharged until their FEV_1 had reached >90% of the subject's baseline value.

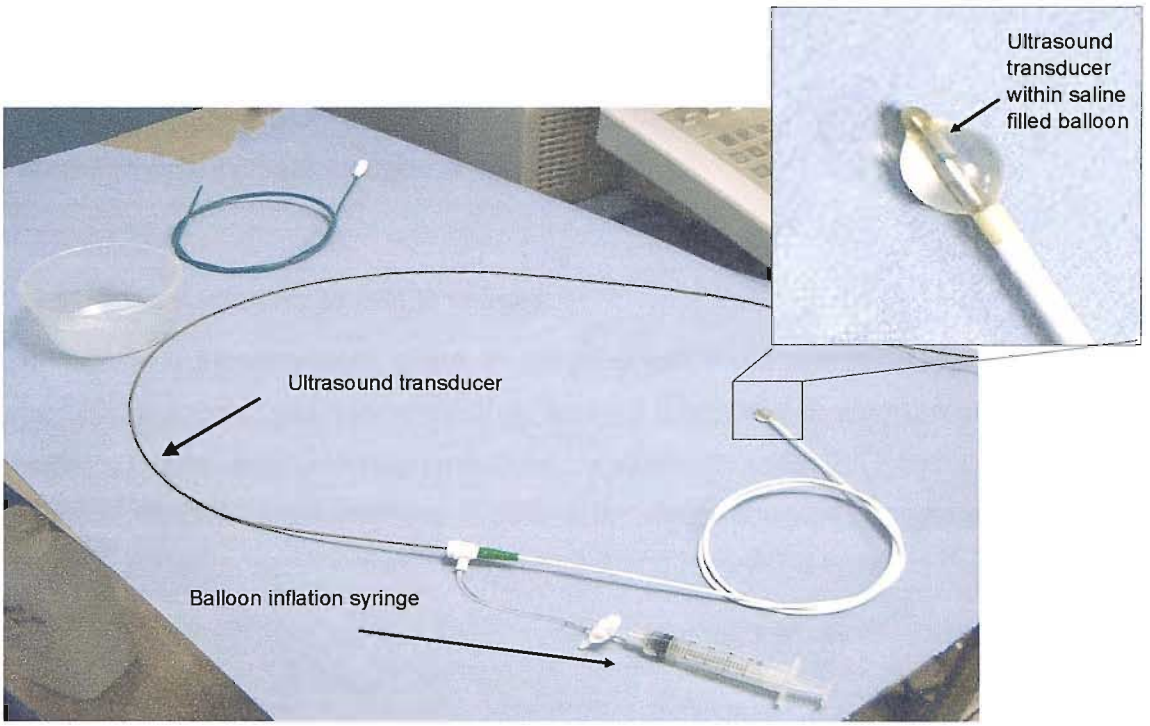
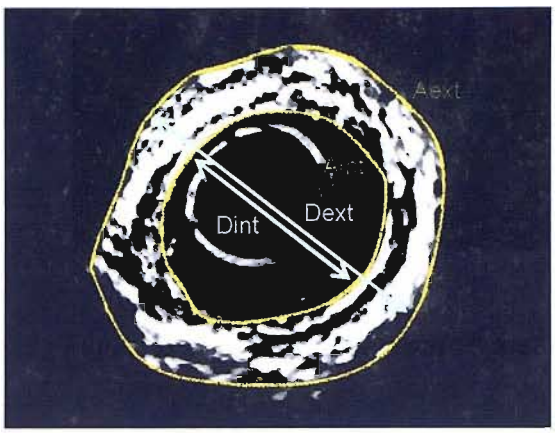


Figure 2-2 EBUS probe and fluid filled sheath

2.9 Measurement of airway wall thickness

EBUS and HRCT images were captured on a computer and stored as Digital Imaging and Communications in Medicine (DICOM) format files. Subject files were anonymised by subject number. Image analysis of both CT and EBUS files was carried out, according to the same protocol, using Osiris 4.18, an image analysis program developed at the University of Geneva (166). The short axis wall thickness to external diameter ratio (T/D) and %wall area (%WA) were calculated from a mean of measurements taken from the 3 EBUS images, or the 3 most appropriately orientated CT slices as described below (see Figure 2-3). All measurements were conducted with the observer blinded to the subject identity and study group.



$$\text{T/D ratio} = \frac{\text{Dext-Dint/2}}{\text{Dext}}$$

$$\%\text{WA} = \frac{\text{Aext-Aint}}{\text{Aext}} \times 100$$

Figure 2-3 EBUS image analysis and calculations

2.9.1 Image analysis of EBUS images

The EBUS ultrasound machine had an attached laptop computer with image capture and DICOM archiving software (WinPax, Medical Diagnostic Systems, Meckesheim, Germany). The Osiris software was manually calibrated using the internal side scale on EBUS images before analysis. Details of the image analysis protocol and an example EBUS image are shown in Figure 2-4 and Figure 2-5 below.

1. Start OSIRIS program
2. GET FILE box will open. Change Drive to the CD drive
3. Double click on folder EBUS IMAGES in the directories list.
4. Single click on PATIENT LIST
5. DICOMDIR box will open
6. Select patient data to be analysed in PATIENTS list on left with one click. The lower half of the screen will show thumbnails of the EBUS images with green boxes round them.
7. Single click on OPEN
8. OSIRIS – DICOMDIR SERIES window will open with the EBUS images in it.
9. Calibrate image series by clicking once on TOOLS and then selecting the "ruler" icon. Move cursor, which now looks like a little ruler, to the left hand end of the horizontal scale bar in the EBUS image, depress the RIGHT mouse button and keeping it pressed draw a line to the right hand end of the scale bar before releasing the button. A CALLIPER DIALOG box will appear. Change "Pixels" to "mm" and type "28" into the left hand box. Click OK.
10. By clicking on the left/right arrows bellow the TOOLS box select the image you wish to analyse
11. Select the irregular region of interest (ROI) tool from the TOOLS menu.
12. Using the mouse click around the inside of the airway. Double click in the small circle at the starting point to link the first and last points.
13. Select SHOW DATA from the OVERLAYS menu at the top of the screen.
14. Record the LENGTH figure in the Pint (internal perimeter) box and AREA in the Aint (internal area) box on the data sheet.
15. Repeat steps 13-15 around the external perimeter of the airway, recording the LENGTH and AREA in the Pext and Aext boxes on the data sheet.
16. Select the ruler icon from the TOOLS menu at the left of the screen.
17. Draw a line across the shortest internal diameter and then the longest internal diameter (not necessary perpendicular).
18. Draw 2 more lines to measure the external diameter at the same sites used for the internal measurements
19. Record these DintSA and DintLA (internal diameter long and short axes) and DextSA/DextLA on the data sheet.
20. Go to 10 to analyse the next image (3 per subject per site)

Figure 2-4 Protocol for EBUS image analysis using Osiris software

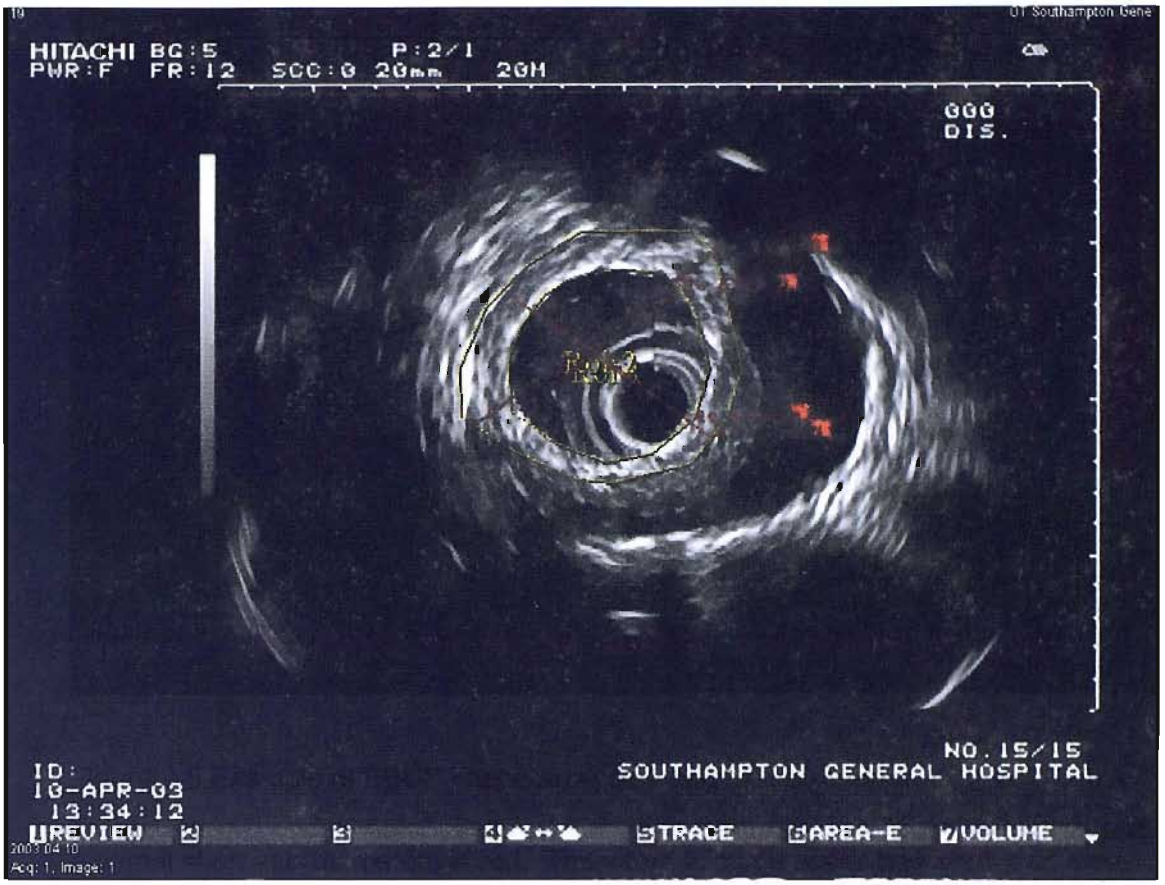


Figure 2-5 Example of EBUS image analysis using Osiris software

Traced internal and external airway perimeters are shown in yellow with internal and external short axis diameters in red. The internal image scale markers are in white along the top and side.

2.9.2 Image analysis of HRCT images

The GE systems CT console was connected to a workstation with CD burning capabilities. Calibration information was automatically included in the DICOM files generated by the CT scanner but the Osiris software was used to specify the window width (1500HU) and level (-450HU) settings before image analysis. After an enlargement of x400 the image analysis procedure used was the same as given in Figure 2-4 for EBUS images. The three images selected for analysis showed the posterior segment of the right lower lobe bronchus cut perpendicular to its axis. An example of the image analysis process of an HRCT image is shown in Figure 2-6.

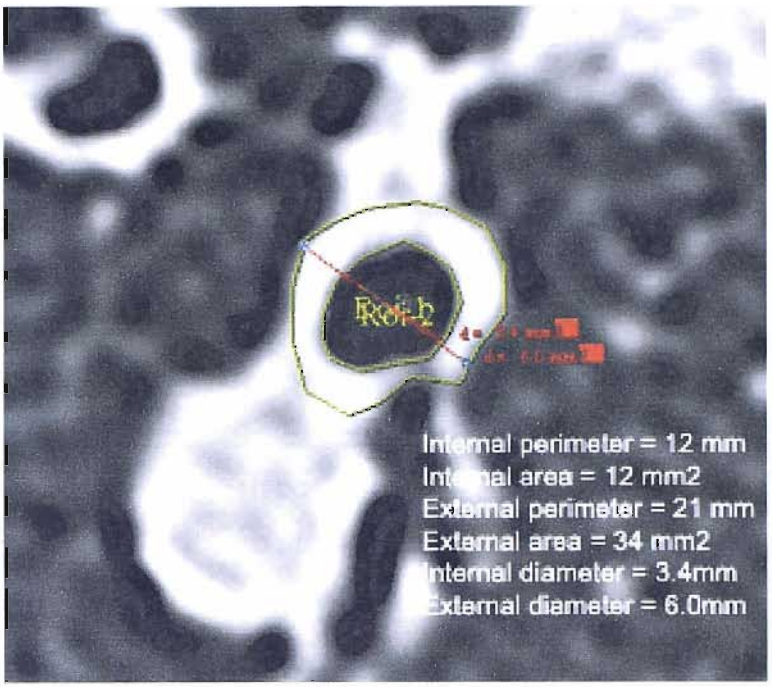


Figure 2-6 Example of HRCT image analysis using Osiris software.

HRCT image (x400) showing the traced internal and external perimeters and measured short axis diameters using the Osiris program. The internal and external perimeters, areas and diameters of the airway walls are automatically calculated by the image analysis program. Calibration data is included with the image header provided by the CT scanner.

Pixilation and blurring of the HRCT image is evident when enlarging to x400 for image analysis. Figure 2-7 shows a comparison between an EBUS and HRCT image of the same airway an at the same magnification.

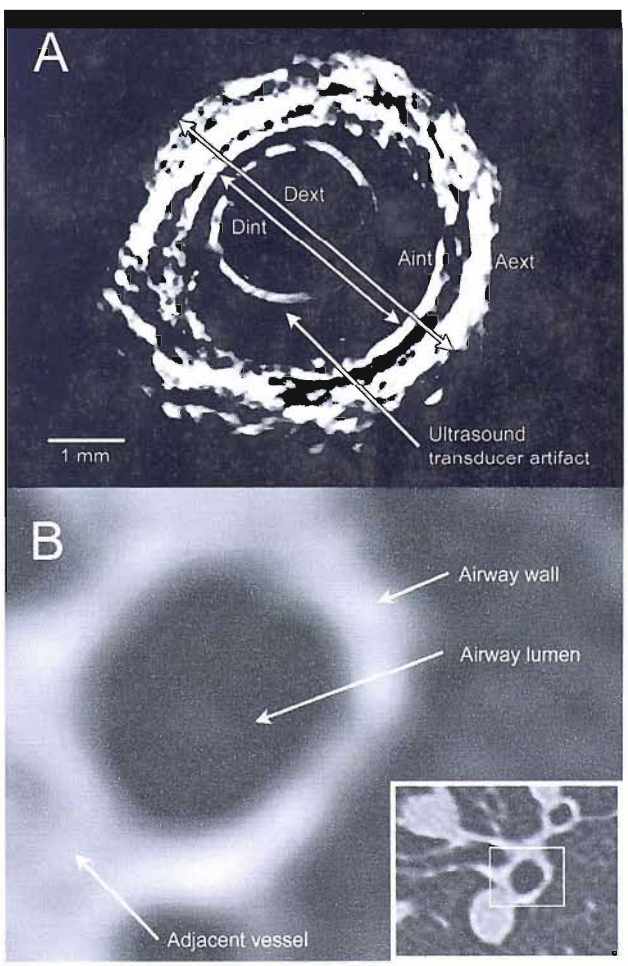


Figure 2-7 Comparison between EBUS and HRCT images of the same airway.

A = EBUS

B = HRCT

2.10 Biopsy processing into glycol methacrylate resin

Endobronchial biopsies were processed into glycol methacrylate (GMA) resin for immunohistochemical staining according to the protocol described by Britten *et al* (167).

The biopsies were fixed in ice-cold acetone containing protease inhibitors overnight at –20°C. The following day the samples were transferred into acetone at room temperature for 15 minutes and then into methyl benzoate for a further 15 minutes. The tissue was then immersed in GMA monomer (solution A) plus 5% methyl benzoate at 4°C for 3 x 2 hour periods at 4°C, the GMA solution being replaced between each incubation. The tissue was finally embedded in GMA resin (prepared by mixing GMA solutions A + B and benzoyl peroxide) in flat-bottomed capsules (Taab, Aldermaston, UK) and left to polymerise overnight at 4°C.

The resin blocks were stored in airtight containers at -20°C. Samples were cut into 2μm sections using a microtome (Ultracut, Leica, Milton Keynes, UK) and floated onto ammonia water (1:500) to be picked up onto 0.01% poly-L-lysine coated glass slides. Two non-contiguous sections were placed on each slide. Toluidine blue staining was performed to assess the best sections for immunostaining. Suitable biopsies had to have an analysable area (excluding muscle, glands and crush artefact) of >0.46mm^2 (168). Once cut, sections were either stained that day or were stored wrapped in aluminium foil at -20°C for up to two weeks in order to preserve tissue antigenicity.

2.11 Immunohistochemistry

GMA embedded sections were stained using a three stage technique. Commercially produced primary antibodies raised in mice, sheep or goats, with specificity for the desired tissue antigens were selected. A biotinolated anti mouse/goat (which also cross reacts with sheep) secondary antibody allowed visualisation of the bound primary antibody, following application of a streptavidin peroxidase enzyme and the appropriate substrate-chromogen solution.

Before performing this technique on bronchial biopsies, the antibodies were optimised on GMA sections of nasal polyp and tonsil. To do this, doubling dilutions of the primary antibodies were used to determine which concentration gave optimal staining of the

features of interest without any non-specific staining. Isotype controls (monoclonal antibodies) or non-immune serum controls (polyclonal antibodies) were used at the same concentration as the test antibody to confirm specificity of the antibody. At the chosen working concentration for the test antibody the matched control had to be negative for this concentration to be used in further experiments on the bronchial biopsies.

When studying the bronchial biopsies negative controls, where the primary antibody was omitted, were included for each specimen studied. None specific binding was inhibited using a culture medium blocking. Endogenous peroxidase activity in the tissues was blocked using a solution containing hydrogen peroxide and sodium azide.

2.12 Immunostaining protocol

Day 1

1. Prepare endogenous peroxidase inhibitor (0.1% sodium azide+0.3% H_2O_2)

2. Cover each biopsy with inhibitor and incubate for 30 mins at room temperature

3. Wash slides with TBS 3 times. Incubating at room temperature for 5 minutes between each wash

4. Drain slides and cover each biopsy with blocking media before incubating for 30 minutes at room temperature

5. Prepare primary antibody dilutions in TBS (see Table 2-1, section 2.11.1 below)

6. Drain slides and apply 200 µL primary antibodies and cover with coverslip.

7. Incubate overnight at room temperature.

Day 2

8. Wash slides with TBS – 3 times, as in step 3 above.

9. Prepare stage 2 antibody dilutions in TBS

(Biotinylated anti-mouse or goat immunoglobulins as appropriate, see Table 2-2, section 2.11.2 below)

10. Drain slides and apply 200µL stage 2 antibodies before incubating for 2 hours at room temperature

11. Prepare streptavidin + biotin + horse radish peroxidase labelled complex

12. Wash slides with TBS – 3 times, as in step 3 above.

13. Drain slides and apply labelled complex before incubating for 2 hours at room temperature

14. Wash slides with TBS – 3 times, as in step 3 above.

15. Drain slides and apply 150µl per slide AEC or DAC as appropriate before incubating for 20 minutes (AEC) or 10 minutes (DAB) at room temperature

16. Rinse slides with TBS then wash in running tap water for 5 minutes

17. Counterstain with Mayer's haematoxylin and blue stain for 30 seconds

11. Wash slides in running tap water for 5 minutes

12. Drain and dry reverse of slides

13. Apply crystal mount and bake in oven at 80°C for 10 - 15 minutes

14. When cool, mount in DPX with cover slip

2.12.1 Primary antibodies

Antibody target (Clone)	Supplier	Type	Chromogen	Dilution	Conc^{n} applied (µg/ml)
Mast cell tryptase (AA1)	Dako Ely, UK	mouse monoclonal IgG1	AEC	1:1000	0.105
Eosinophils (EG2)	Pharmacia & Upjohn, Uppsala, Sweden	mouse monoclonal IgG1	AEC	1:500	2
Collagen I (col-1)	Abcam Cambridge, UK	mouse monoclonal IgG1	DAB	1:400	14.5
Collagen III (3G4)	Chemicon Temecula, USA	mouse monoclonal IgM	DAB	1:150	30
Collagen V (-)	Chemicon Temecula, USA	goat polyclonal	DAB	1:80	5
αSMA (1A4)	Sigma Gillingham, UK	mouse monoclonal IgG2	DAB	1:40,000	0.055
Fibronectin (EP5)	Cymbus biotech Chandlers Ford, UK	mouse monoclonal IgG1	DAB	1:20	5
Biglycan (-)	Biogenesis Poole, UK	sheep polyclonal	DAB	1:50	488
Decorin (-)	Biogenesis Poole, UK	sheep polyclonal	DAB	1:50	230
Tenascin (TN2)	Dako Ely, UK	mouse monoclonal IgG1	DAB	1:60	11
Perlican	Zymed San Francisco, USA	mouse monoclonal IgG1	DAB	1:200	2.5

Table 2-1 Primary antibodies

2.12.2 Secondary antibodies

Antibody type	Supplier	Dilution*
rabbit à mouse (biotinylated)	Dako, Ely, UK	1:300
rabbit à goat (biotinylated) (cross reactivity with sheep)	Jackson ImmnoResearch Soham, UK	1:10,000

Table 2-2 Secondary antibodies * concentrations not available

2.12.3 Reagents for GMA processing and staining

- Acetone -containing protease inhibitors
  2mM phenylmethylsulphonyl fluoride with 20 mM iodoacetamide
  Sigma, Poole, UK

- Methyl benzoate
  Merck, Lutterworth, UK.

- GMA resin (Solutions A + B)
  JB4 embedding kit, Park Scientific, Northampton, UK.

- Endogenous peroxide inhibitor
  5ml 0.1% sodium azide + 50µl 30% H_2O_2, per tray of 24 slides

- Tris buffered saline (TBS)
  Mix sodium chloride (80g) + Tris (6.05g) + 1M HCL (38ml) in 1L reverse osmosis purified water (ROW). Adjust to pH 7.65 and make up to 10L with ROW.

- Blocking medium
  Dulbecco's modified Eagles medium 80mls (Sigma, Poole, UK) + fetal calf serum 20mls (PAA Biologicals, Consett, UK) + bovine serum albumin 1g (Sigma, Poole, UK). Keep at -20°C until required.

- Tris/HCL buffer
  Mix 0.2M Tris (12mls) + 0.1M HCL (19mls) + ROW (19mls) and adjust to pH 7.6 with TBS

- Steptavidin + biotin + horse radish peroxidase
  1µl of A + 1µl of B + 198µl of Tris/HCL per slide.
  Incubate at room temperature for at least 30 minutes to complex before use

- AEC/DAB chromogen kits
  Merarini Diagnostics, Wokingham, UK

2.13 Measurement of RbM thickness

Reticular basement membrane thickness was assessed using a Zeiss microscope with high resolution digital video camera and KS400 (Image Associates) software. Verified calibration files were loaded for each session and microscope settings standardised for each staining run - objective lens, automatic white balance, light level and condenser/iris settings. Using x 400 magnification areas of undamaged and well orientated RbM were visualised on 2 sections from each biopsy after staining with toluidene blue. A digital image of each view was captured and the margins of the RbM manually traced. The results were expressed as mean thickness in μm and biopsies with least 1mm of appropriately orientated RbM were selected (168). The subject group and identity were blinded to the investigator.

2.14 Analysis of immunohistochemistry stained sections

Collagen/proteoglycan staining in the lamina propria was assessed using the same hardware described in section 2.13. The microscope and KS400 software settings were standardised for each staining run – objective lens, automatic white balance, light level and condenser/iris settings. Low power images of each biopsy section were digitally captured and printed out, to allow identification of appropriate areas of the submucosa for study, excluding epithelium, RbM, blood vessels and glands. After manual delineation of the pre-identified submucosal regions, areas of positive staining were identified according to their red, green and blue colour balance. The results were expressed as a % positive staining of the total submucosal area available on both sections of each biopsy. Non specific binding was avoided by use of a blocking medium during processing and selection of a primary antibody concentration that gave no staining on test tissue samples of tonsil and nasal polyp.

Eosinophil density in the submucosa was assessed by manual counting of immuno-stained nucleated cells with the results expressed as cells/mm^2 of submucosa, again excluding epithelium, RbM, blood vessels and glands. αSMA staining was assessed using the image analysis system described above and the same macro but results were expressed as % staining of the whole biopsy area. The subject group and identity were blinded to the investigator.

2.15 Statistical analysis

Statistical analysis was performed using SPSS Version 11.5 (SPSS Inc, USA). The data were summarised using the mean and standard deviation for parametric data and median and range for non-parametric data. The intra/inter site and intra/inter observer variability were compared using the methods described by Bland and Altman (146) and intraclass correlation coefficients (ICC) calculated where appropriate. EBUS and HRCT measurements of T/D and %WA were also compared using Bland and Altman's methods, and mean within site coefficients of variation (COV) calculated for each airway parameter. A p value of < 0.05 was taken to indicate statistical significance.

Chapter 3: Validation of EBUS for measurement of airway wall thickness

3 Validation of EBUS for measurement of airway wall thickness

3.1 Introduction

Where EBUS is used clinically to detect tumour invasion this is indicated by a disruption of the peri-cartilage echo pattern. In these circumstances it does not matter whether the balloon sheath causes compression of the more internally placed epithelial and submucosal tissues (169, 170). If the technique is to be validated for assessment of non-malignant conditions, such as airway wall thickening in asthma, possible distortions of these structures due to balloon inflation become important. The balloon sheath, whilst essential when the EBUS probe is used *in vivo*, may be dispensed with in *ex vivo* experiments where material may be completely immersed in fluid to provide the sonic coupling necessary to achieve a 360° image.

3.2 Aims

Before the *in vivo* study of human airways a validation study was conducted of explanted sheep airways to answer the following questions:-

1. Does inflation of the EBUS balloon sheath modify the airway internal diameter, thickness or wall area in explanted tissue?

2. Can measurements of AWT and area taken from EBUS images be correlated with those obtained by morphometric analysis of paraffin embedded specimens from the same area?

3.3 Methods

3.3.1 Study procedure

Four adult female Welsh mountain sheep were sacrificed with intravenous sodium pentabarbitone (150 mg/kg) and a post mortem was performed. The animals were part of a research project by another group in the University of Southampton, School of Medicine who had no requirement for the lung tissue, which would otherwise have

been discarded. Both lungs and the heart were removed en-bloc and six segmental cartilaginous airways of 4-5mm internal diameter and 8-10mm length were dissected out from each animal and placed in phosphate buffered normal saline (PBS). Secretions, blood and mucous within the airway were removed by gentle irrigation with PBS. Each bronchus was held vertically by insertion of a 22G needle through peripheral adventitial tissue away from the area to by studied, before immersion in PBS. The 20MHz ultrasound probe (PL2220-20, Hitachi medical systems, Japan) with its latex balloon sheath was held centrally within the airway and two images captured electronically (see Figure 3-1). The balloon sheath was inflated twice, to the minimum amount required to cause contact with the airway wall and obtain a 360° image, and again images were recorded. Following the ultrasound imaging the bronchi were fixed for at least 48 hours in 10% neutral buffered formalin and the central 3-4 mm cut out to form rings for embedment in paraffin wax. Four micron sections were cut and stained with Haematoxylin and Eosin (H & E) for morphometric analysis.

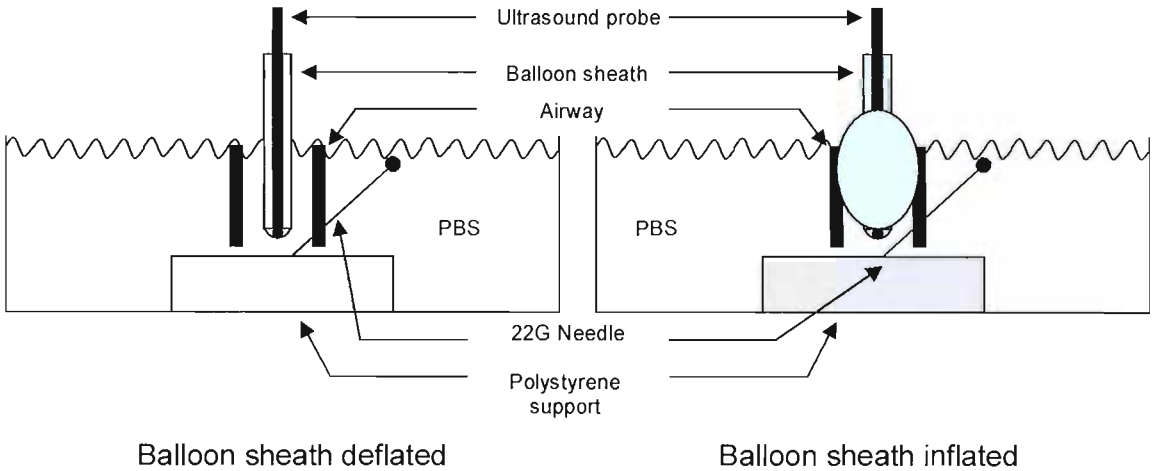


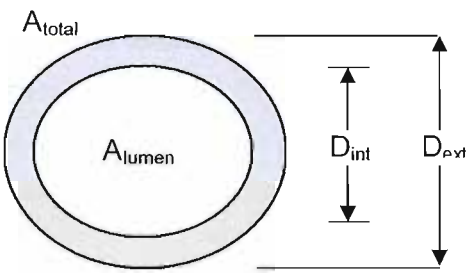
Figure 3-1 Equiment used in sheep explant study

The EBUS probe is held vertically within the submerged airway and images captured with the balloon inflated and deflated

3.3.2 Image analysis

The ultrasound images generated were captured by computer and stored as Digital Imaging and Communications in Medicine (DICOM) format. Image analysis was carried out using Osiris 4.18, the same program used in the *in vivo* work described in chapter 2 (166). Light microscopy was carried out using a Zeiss microscope and Axiocam

camera with image analysis of the TIF files generated, by Image J (US National Institute of Health). For each of the three experimental conditions - EBUS without balloon inflation, EBUS with balloon inflation and H & E stained tissue; the same image analysis protocol was followed. The short axis wall thickness (WT) and internal diameter (D) were measured directly using electronic callipers and the wall area (WA) was determined by subtraction of the luminal from the total airway area (see Figure 3-2).



D_{int} = Internal diameter (short axis)

D_{ext} = External diameter (short axis)

A_{total} =Total area

A_{lumen} =Lumenal area

Internal diameter (D) (mm) $= D_{int}$

Wall thickness (WT) (mm) $= (D_{ext} - D_{int})/2$

Wall area (WA) (mm^2) $= A_{total} - A_{lumen}$

Figure 3-2 Image analysis for sheep explant study

3.3.3 Statistical analysis

The data were summarised using the mean and standard deviation for each airway parameter. The EBUS measures of internal diameter, wall airway thickness/area with and without the balloon inflation were assessed using the methods of Bland and Altman (146). The mean and standard deviation of the differences between methods, limits of agreement (+/- 2 SD) and intraclass correlation coefficients were calculated. The same techniques were used to assess agreement between EBUS and morphometric measurements taken from the H&E stained sections. A p value of < 0.05 was taken to indicate statistical significance.

3.4 Results

3.4.1 EBUS images

Ultrasound images of the segmental cartilaginous airways revealed five distinguishable layers (Figure 3-3). The first layer (hyperechoic) is a marginal or interface echo formed where the balloon sheath/PBS is in contact with the epithelium. The second layer (hypoechoic) corresponds largely to the submucosal tissue. The third layer (hyperechoic) is a marginal echo formed by the inner surface of the bronchial cartilage. The fourth (hypoechoic) layer is the cartilage itself. The fifth (hyperechoic) layer is the marginal echo formed by the change in echodensity at the outer cartilage edge. The ultrasound bands corresponding to the various histopathological structures have been previously confirmed by investigators who have inserted needles, that may be visualised on the ultrasound image, into the various layers.(160, 162).

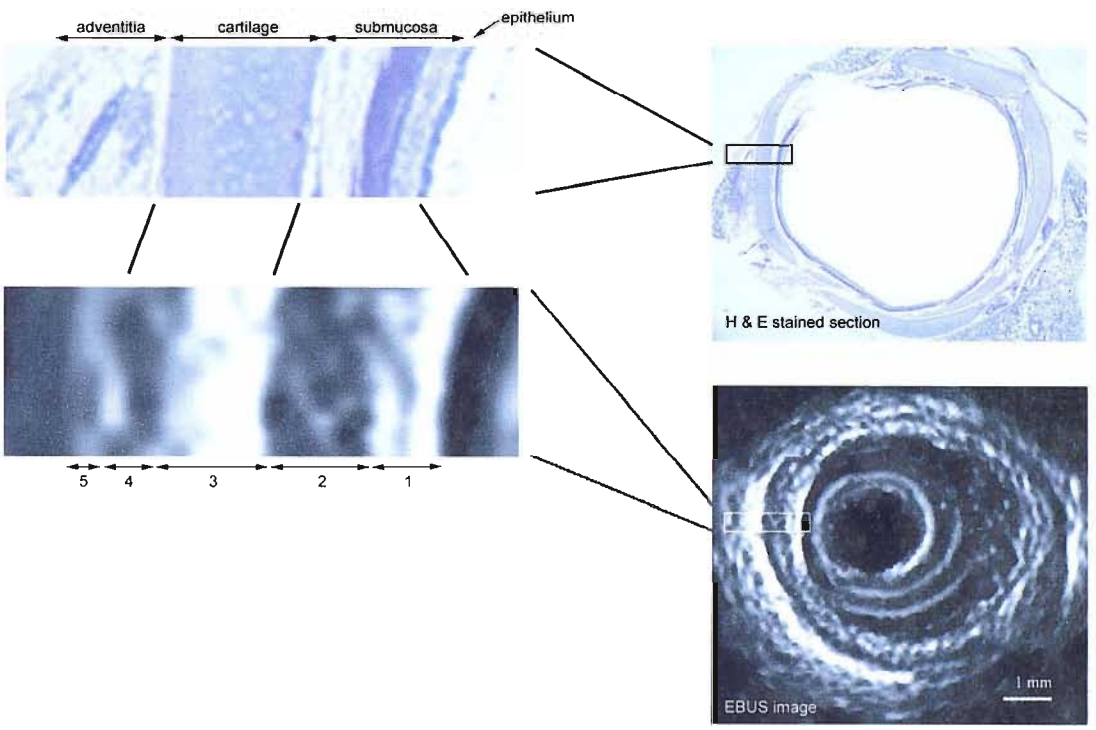


Figure 3-3 EBUS images from sheep explant model

Comparison between EBUS image of a segmental airway (with balloon sheath inflated) and an H & E stained section from the same site. 1 - First band (hyperechoic), 2 - second band (hypoechoic), third band (hyperechoic), forth band (hypoechoic), fifth band (hyperechoic)

3.4.2 Airway characteristics

24 airway were studied, six from each of four sheep. Details of the airways are summarised in Table 3-1.

Airway parameter	EBUS nb	EBUS b	H & E
D	4.3 (0.8)	4.2 (0.8)	**4.5 (0.8)**
WT	1.5 (0.3)	1.6 (0.3)	**1.2 (0.3)**
WA	**27.8 (7.3)**	**31.1 (8.8)**	**24.1 (8.6)**

Table 3-1 Sheep explant model, airway characteristics

Results expressed as mean (SD). nb = no balloon sheath inflation, b = balloon sheath inflation, D = short axis internal diameter (mm), WT = short axis wall thickness (mm), WA = total wall area (mm^2)

3.4.3 Repeatability

Each airway was measured twice using EBUS without balloon inflation and twice following inflation. Bland–Altman plots were constructed for all the comparisons – EBUS without vs. with the balloon and EBUS without the balloon vs. morphometry these are shown in Figure 3-4. These illustrate the average difference between the mean of the two measurements (of D ,WT or WA) without/with the balloon against the difference between them (without minus with) and show the mean difference in internal diameter (D) to be only 0.08mm, wall thickness difference to be 0.06mm and wall area difference to be 3.29mm^2. The random scatter demonstrates that there is no systematic relationship between the difference and the mean.

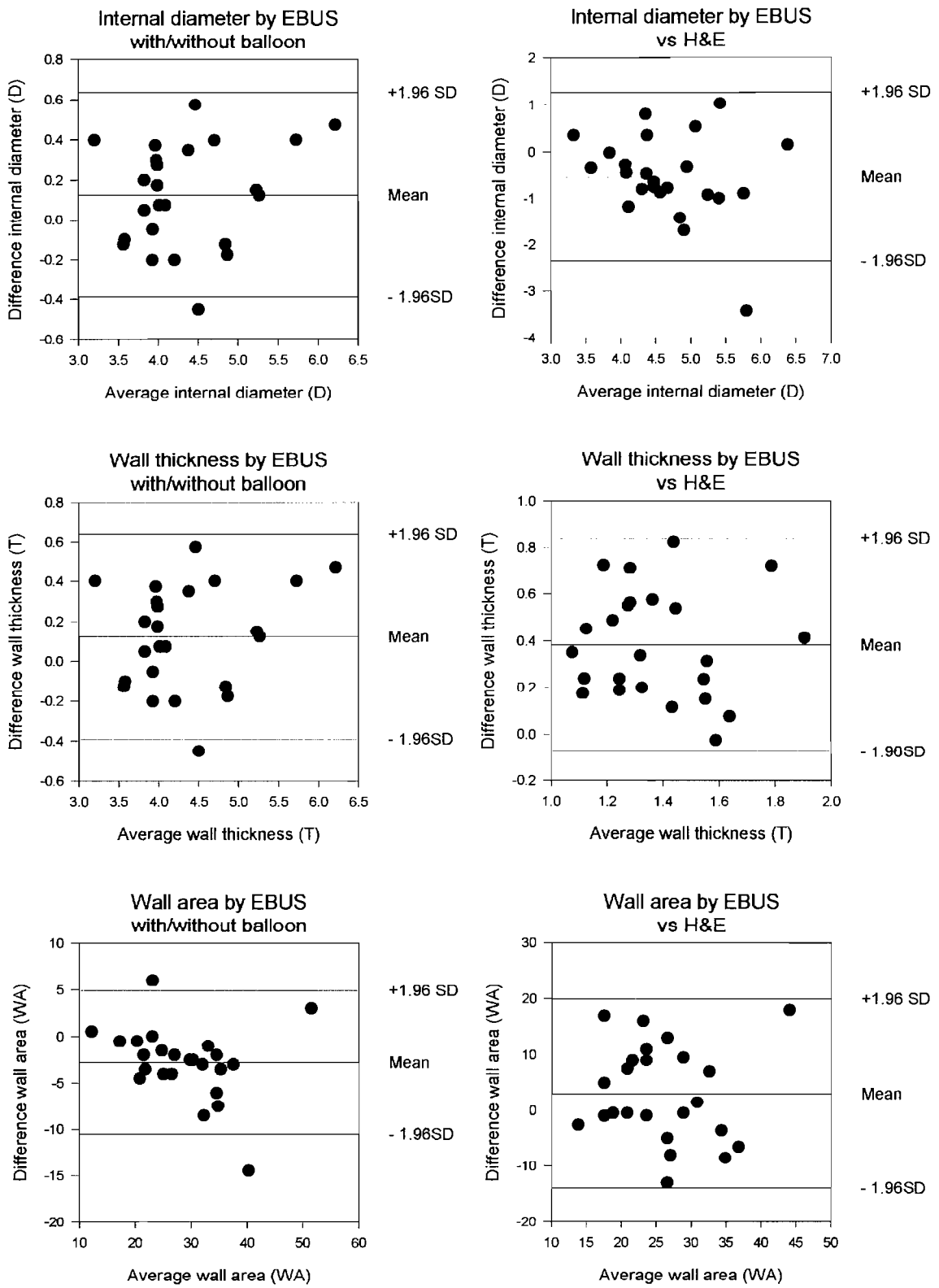


Figure 3-4 Bland-Altman plots in sheep explant study

The plots show the airway parameters derived from EBUS images without vs. with the balloon (left column) and EBUS without the balloon vs morphometry (right column)

A summary of the results, intraclass correlation coefficients and significance values for each comparison are shown in Table 3-2. Measurements using EBUS with and without inflation of the balloon sheath provide comparable results with respect to D (intraclass correlation coefficient (ICC) 0.97, p<0.001), T (0.88, p<0.001) and WA (0.95, p<0.001). When morphometric measurements of the H&E images are compared with EBUS the limits of agreement are greater and ICC values poorer though some just achieve significance - D (ICC 0.51, p<0.05), T (0.24, p=n/s) and WA (0.67, p<0.05).

	Mean difference	**SD of difference**	**Limits of agreement***	**ICC**	**Significance**
EBUS nb vs b					
D	0.08	0.29	-0.49 to 0.66	0.97	**p<0.001**
WT	-0.06	0.17	-0.40 to 0.27	0.88	**p<0.001**
WA	-3.29	3.46	-10.21 to 3.62	0.95	**p<0.001**
EBUS nb vs H&E					
D	-0.25	0.95	-2.15 to 1.66	0.51	**p<0.05**
WT	0.32	0.34	-0.36 to 1.01	0.24	**p = n/s**
WA	3.52	7.7	-12.07 to 19.11	0.67	**p<0.05**
EBUS nb report 1 vs report 2					
D	0.13	0.52	-0.92 to 1.17	0.90	**p<0.001**
WT	-0.07	0.36	-0.80 to 0.66	0.46	**p = n/s**
WA	-1.29	4.95	-11.19 to 8.60	0.87	**p<0.001**
EBUS b report 1 vs report 2					
D	-0.10	0.33	-0.76 to 0.57	0.95	**p<0.001**
WT	-0.04	0.25	-0.54 to 0.47	0.78	**p<0.001**
WA	**0.79**	**6.20**	**-13.19 to 11.61**	**0.86**	**p<0.001**

Table 3-2 Sheep explant study summary of results

Assessment of agreement using different techniques. nb = no balloon sheath inflation, b = balloon sheath inflation, D = short axis internal diameter (mm), WT = short axis wall thickness (mm), WA = total wall area (mm^2), * 95% confidence limits, ICC = intraclass correlation coefficient

3.5 Discussion

In this preliminary study it has been demonstrated that inflation of the latex balloon sheath around the EBUS transducer does not significantly modify the internal diameter of the airway, the wall thickness or the wall area. Significant correlation coefficients were also seen between these parameters as measured by EBUS with and without inflation of the balloon sheath. Morphometric measurements of paraffin sections showed less consistent agreement with those derived from EBUS image analysis.

3.5.1 Internal airway diameter

The close association between EBUS measurements taken with/without inflation of the balloon is dependent on inflation being discontinued as soon as contact with the airway wall is achieved as over inflation would undoubtedly lead to airway distortion. The short axis internal diameter was used in the analysis as it has previously been shown to be less influenced by the effects of partial voluming. This is the apparent increased diameter and wall thickness seen in the long axis dimension when an airway is cut obliquely rather than at right angles (145). In our work care was taken to orientate the airway to avoid an oblique cut and hence provide a circular image for analysis.

3.5.2 Wall thickness

Ultrasound echoes detect abrupt changes in density most clearly and the inflation of the balloon gives the internal margin of the airway a more distinct outline than the saline/wall interface seen when the balloon is deflated. Ultrasound images recorded with the balloon inflated demonstrated a slightly greater AWT, of approximately 0.5mm, than when it was not inflated, though this difference did not reach significance. Clearly when the latex balloon sheath is in contact with the airway its thickness is included in the measured wall thickness, however the sheath is only 0.05mm thick and this does not account for all the observed difference. A more likely explanation is that the sheath/wall contact allows the inner airway wall to be identified more readily during image analysis. Ultrasound echoes are most apparent in regions of rapidly changing density and the balloon sheath clarifies the density change between that seen in the lumen and the bronchial wall. The result of this is seen in the improved intra airway repeatability of EBUS when the balloon is employed.

3.5.3 Wall area

Wall area is a function of the airway diameter and thickness when the section analysed is circular. In this study care was taken to orientate the sections and avoid branch points to give a circular image and therefore as expected the results comparing wall area with and without balloon inflation confirm a close correlation.

3.5.4 Morphometric analysis

Although statistically significant correlations were observed between the internal diameter and wall area measured using ultrasound and equivalent measurements derived from morphometry, these correlations were less strong and the measurements of AWT failed to reach significance. In comparison with ultrasound derived measurements the morphometric analysis showed an increase in the internal diameter (of approximately 5%) and a decrease in wall thickness (21%) and wall area (13%). This tissue shrinkage occurs during the processes of fixation and dehydration and the degree to which it occurs depends on the cellular components of the tissue being fixed (130). Airway wall sections contain variable ratios of cartilage and less rigid structures such as smooth muscle, glandular and vascular tissues. Figure 3-3 clearly shows the greater shrinkage of the epithelial/submucosal layer when compared with that seen in the cartilage layer. It is highly probable that the differential shrinkage of these tissues lead to the discrepancies seen between ultrasound and morphometric measures and the latter cannot be taken as a gold standard (130, 171, 172).

3.5.5 The EBUS banding pattern

There is widespread disagreement in the published literature on the interpretation of the ultrasound banding pattern seen with the EBUS probe (158, 160-162, 173). There is agreement that where cartilage is present at least three clear hyperechoic bands are seen (Figure 3-3), at the luminal margin and either side of the cartilage layer. Distal to the segmental airways cartilage exists as irregular plates and incomplete rings in a highly variable distribution, which gives rise to a great variation in EBUS airway appearances (174).

The airway structures corresponding to the hyperechoic bands seen on an ultrasound image were identified by Kurimoto *el al* who inserted pins using a stereoscopic microscope into surgically resected human tracheal tissue and went on to identify the

position of these pins on a corresponding ultrasound image (160). The same research group submerged in water whole resected lungs, from patients undergoing resection for lung cancer and were able to describe the ultrasound appearances of normal bronchial wall tissue and that invaded by malignant tissue for the first time.

An *in vivo* study by Tanaka *et al* patients with a variety of primary and secondary intra thoracic malignancies underwent EBUS to determine tracheo bronchial invasion (161). Images were compared where possible with subsequent resected tissue. An accuracy of 93.3% was claimed but in only 20/35 cases was histological material obtained to confirm the degree of invasion and only 9/35 had bronchogenic carcinoma. The remaining cases involved extrabronchial wall invasion where the tumour extended out of the field of view. A key point emerged and this was that the clearest identifiable reference marker was the hyperechoic cartilage in the wall and disruption of this was suggested invasion as malignant tissue appeared hypoechoic.

Another Japanese study by Baba *et al* again examined tumour invasion into the bronchial wall (162). EBUS was performed on 21 patients in whom bronchoscopically visible tumour was seen prior to their pneumonectomy/lobectomy and on a further 40 patients on the resected specimen. Ultrasound images and histology were compared with agreement on invasion (again referenced to the cartilage layer) and cartilage thickness. Perhaps not surprisingly the ultrasound images were clearer in immersed resected specimens than *in vivo*. In the discussion the authors note that due to the increased thickness of the first interface echo, which includes the latex balloon to tissue interface as well as the epithelium, it is not possible to measure the thickness of the latter.

The first hyperechoic band, formed at the interface of the epithelial surface and the airway lumen, is significantly broader than the epithelium alone (175). Previous work on the gastrointestinal tract (153, 176, 177) and human airways (160) has confirmed this finding and until recently it is not possible to quantify individual airway wall structures with current EBUS technology. A recent report from a Swiss group describes reproducible measurements of individual airway layers but correlation with specific structures is not possible as histological samples were not taken (178).

3.5.6 Conclusions

This study demonstrates that the 20MHz radial EBUS probe can be used to assess total airway thickness and wall area in the cartilaginous airways and that inflation of a water filled latex sheath over the transducer does not significantly influence these measurements. Comparison of ultrasound images with samples embedded in paraffin and stained for morphological analysis reveals that histopathological measures are hampered by tissue shrinkage during processing and as such EBUS gives a more accurate reflection of airway wall dimensions.

Chapter 4: Airway wall thickening in asthmatic and control subjects

4 Airway wall thickening in asthmatic and control subjects

4.1 Introduction

An increase in total AWT has been demonstrated in asthmatics compared to control subjects both in studies of post mortem tissue (72, 75-77, 86) and HRCT imaging *in vivo* (136, 138-140) as outlined in chapter 1. To my knowledge there is no published data on the assessment of AWT using endobronchial ultrasound.

4.2 Aims

1. To compare AWT in asthmatic and control subjects using both EBUS and HRCT

2. To compare measurements of AWT assessed using EBUS and HRCT in the same group of subjects

3. To assess inter and intra observer variability in AWT as measured by EBUS in a subset of control subjects

4. To assess within subject inter lobe variability and repeatability measurements of AWT in the same lobe

4.3 Methods

4.3.1 Study protocol

Asthmatic and control subjects were selected, recruited and physiologically characterised according to the methods described in chapter 2. Subjects attended for 3 scheduled study visits as shown previously in Figure 2-1 (page 42). Images obtained by EBUS and HRCT were analysed according to the protocol described previously. A subset of the control group underwent additional EBUS image acquisition from two other sites within their lungs, in right middle (RML) and left lower lobe (LLL) as described below. All subjects had EBUS measurements from the posterior basal segment bronchus of right lower lobe (RLL).

4.3.2 Inter observer variability in EBUS image analysis

All airway measurements were conducted by myself, blinded to the patient identity and group. Inter observer variability was assessed by having 2 observers (myself and a radiology specialist registrar) conduct the same measurements independently on a subgroup of 10 of the control subjects.

4.3.3 Intra observer variability in EBUS image analysis

In order to assess intra observer variability, the same image analysis measurements were repeated on the stored DICOM files after an interval of at least 6 weeks on a subgroup of 10 control subjects. The first series of airway measurements was used in all other analyses.

4.3.4 Within subject inter lobe variability of airway wall thickness using EBUS

To assess the variation in wall thickness between different airways within the same subject a subgroup of 11 control subjects underwent additional imaging of the lateral segment bronchus of middle lobe and posterior basal segment bronchus of left lower lobe.

4.3.5 Repeatability of same lobe measurements of airway wall thickness

Repeatability of AWT measurements within the 3 right lower lobe measurements was calculated for all asthmatic and control subjects.

4.3.6 Statistical analysis

Subject characteristics and AWT parameters were summarised using as mean (SD) or median (range) as appropriate. Comparisons between controls and asthmatics using EBUS or HRCT were performed using the Mann Whitney U test. Agreement between HRCT and EBUS measurements was assessed using the methods described by Bland and Altman (146) and plotted graphically with calculation of intraclass correlation coefficients (ICC). Intra observer and Inter observer reproducibility was also assessed using similar methods. Within subject inter site variability of EBUS was assessed by calculating the coefficient of variation (COV) for each airway parameter. The mean intra site COV was also calculated as 3 measurements were made at each site. A p value of < 0.05 was taken to indicate statistical significance.

4.4 Results

4.4.1 Subject characteristics

CT and EBUS data on AWT was available on 26 subjects. Details of their baseline characteristics are shown in Table 4-1.

Subjects	Asthmatics	Controls
Number	12	14
Duration of asthma (years)	22 (13)	-
Age (years)	37 (14)	40 (3)
Inhaled steroids (7 subjects) (BDP mcg/day)	300 (0 – 2000)	-
FEV_1 (baseline, %predicted)	90 (13)	107 (10)
Reversibility to β_2 agonists (%)	12 (12)	0 (5)
PC_{20} (Histamine, mg/ml)	1.1 (0.1 – 8.00)	>8

Table 4-1 Subject characteristics

Data are shown as mean (SD) or median (range)
BDP = Equivalent total daily dose of Beclomethasone Diproprionate

4.4.2 EBUS measurements of airway wall thickness

Box plots of the AWT parameters T/D and %WA in asthmatics and controls using EBUS are shown in Figure 4-1. The boxes represent the median and 25 – 75th percentile range whilst the whiskers show the 5 – 95th percentile ranges. Individual cases are marked as circles and all outliers are included. The significance values shown were generated using the Mann Whitney U test and confirm that AWT, measured using EBUS, is increased in asthmatics over control subjects.

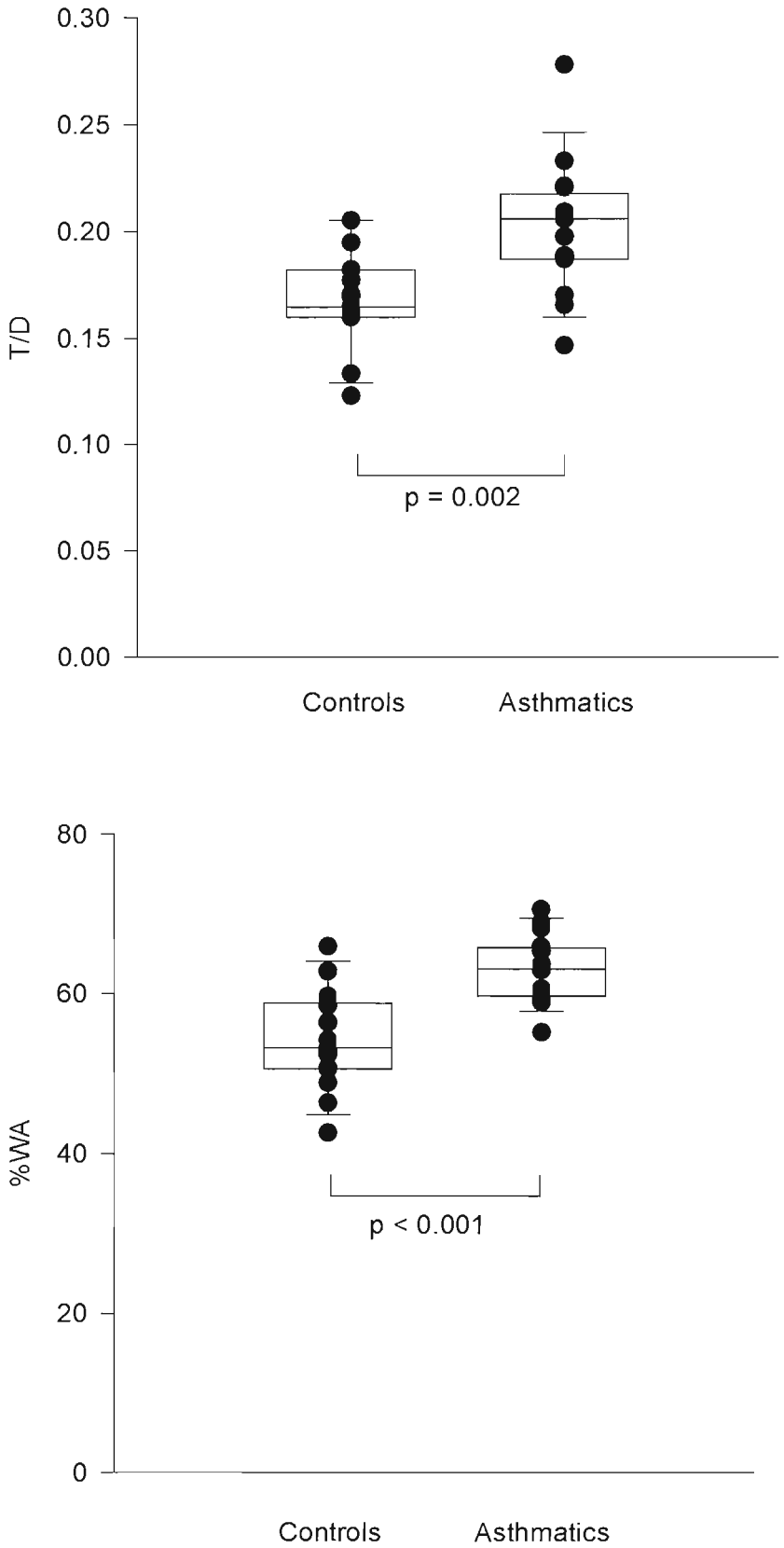
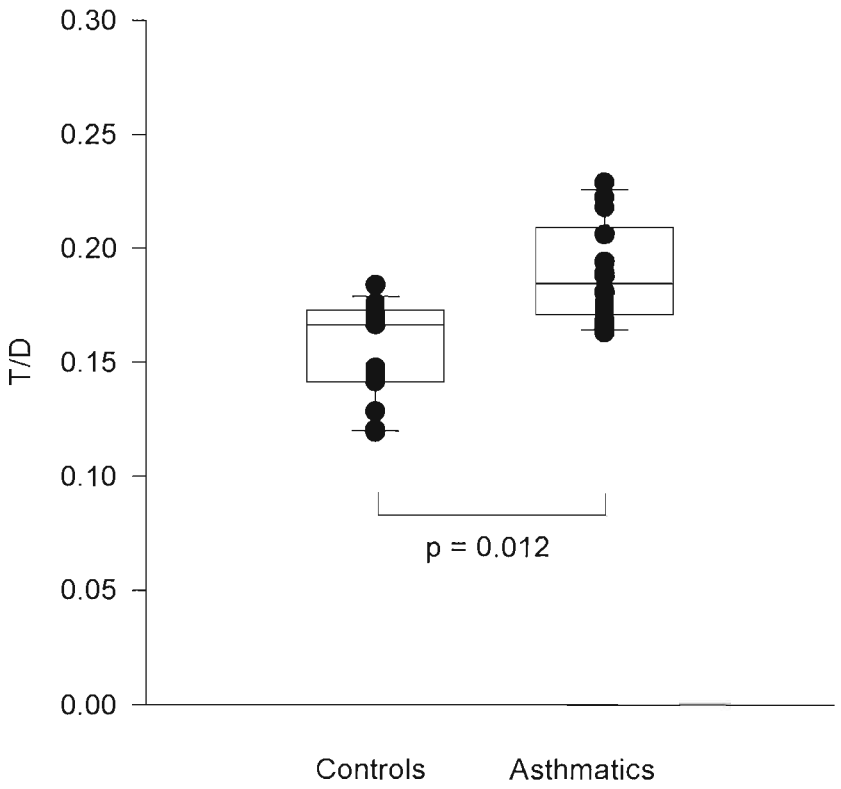


Figure 4-1 AWT in asthmatics and control subjects using EBUS

4.4.3 HRCT measurements of airway wall thickness

Box plots of the AWT parameters T/D and %WA in asthmatics and controls using HRCT are shown in Figure 4-2. The significance values shown were generated using the Mann Whitney U test and confirm that AWT, measured using HRCT, is increased in asthmatics over control subjects.



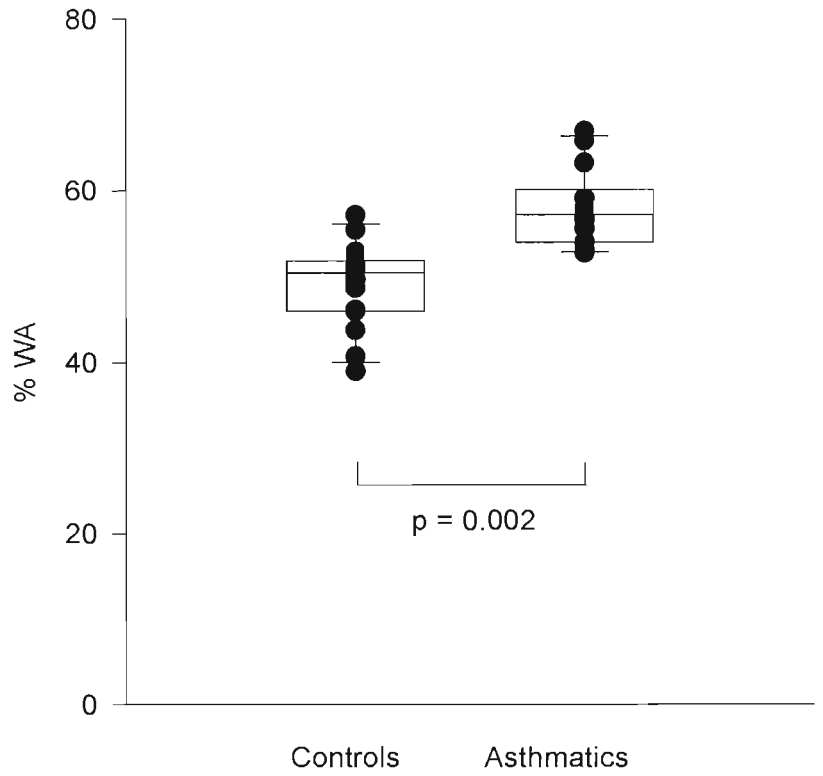


Figure 4-2 AWT in asthmatics and controls using HRCT

4.4.4 EBUS *vs.* HRCT measurements of airway wall thickness

Bland and Altman plots showing the level of agreement between AWT parameters T/D and %WA measured in asthmatics and controls using EBUS and HRCT are shown in Figure 4-3. In each case the mean difference was close to zero and there was no obvious relation between the measurement error and airway parameter. The intraclass correlation coefficients (ICC), showing levels of agreement between measurements using EBUS and HRCT, are shown in Table 4-2.

Airway parameter	ICC value	Significance
T/D	0.68	$p < 0.001$
%WA	0.81	$p < 0.001$

Table 4-2 Intraclass correlation coefficients between EBUS and HRCT measurements

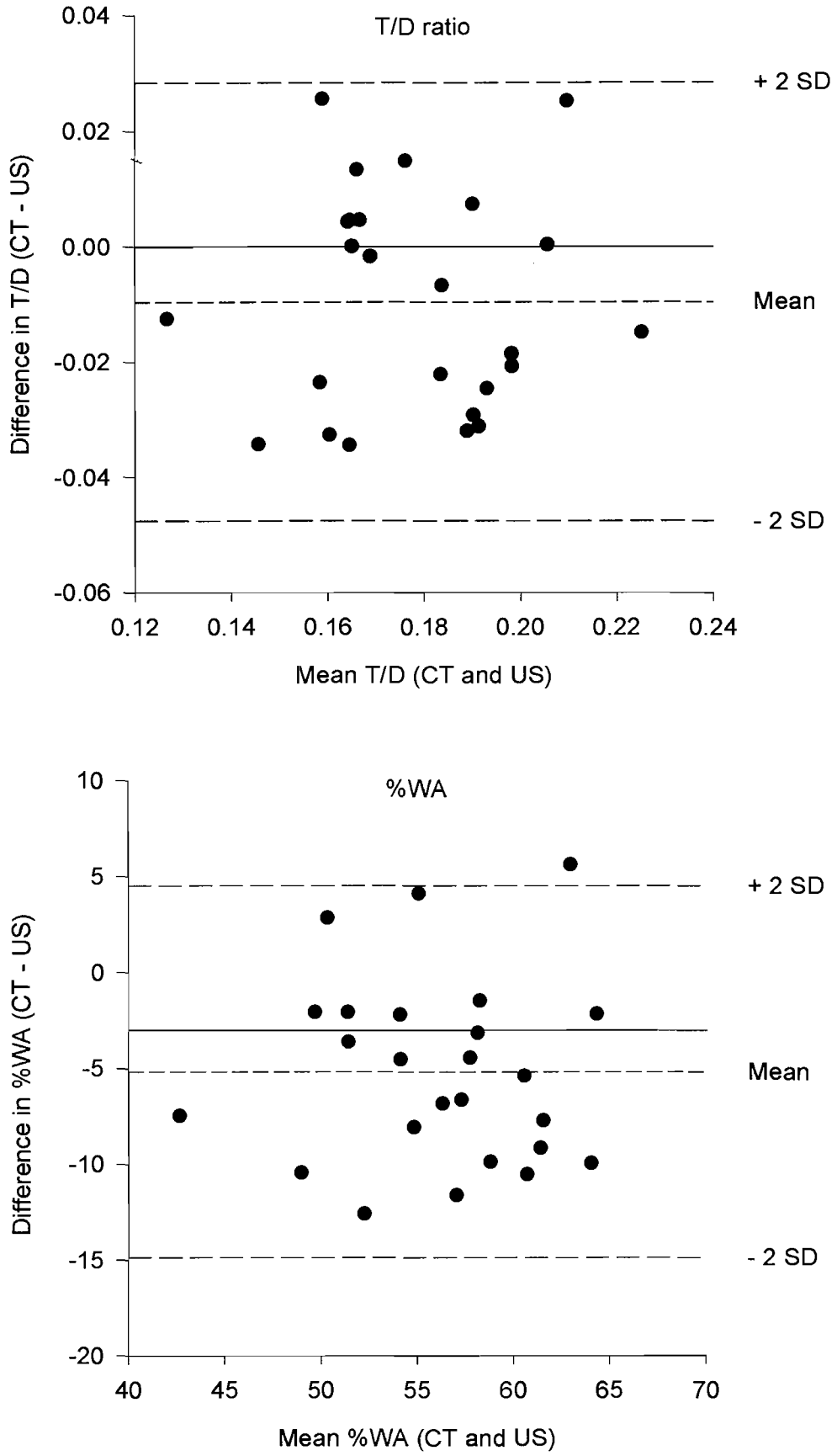


Figure 4-3 Bland-Altman plots showing agreement between EBUS and HRCT

4.4.5 Inter observer variability in EBUS image analysis

Bland and Altman plots showing the mean *vs.* difference between the AWT parameters obtained from image analysis by 2 independent observers are shown in Figure 4-4. In each case the mean difference was close to zero and there was no obvious relation between the measurement error and airway parameter.

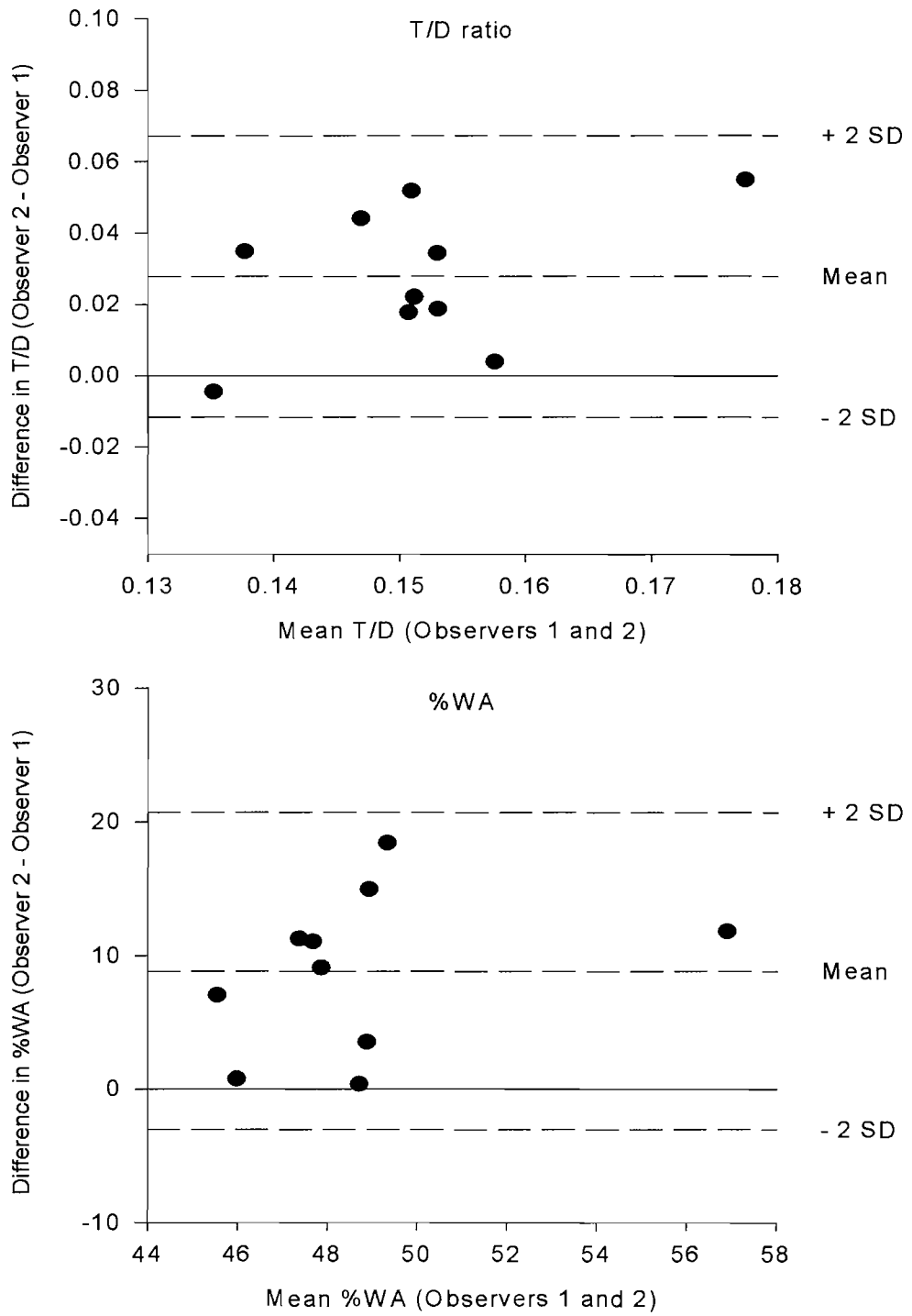


Figure 4-4 Bland-Altman plots showing inter observer agreement using EBUS

4.4.6 Intra observer variability in EBUS image analysis

Bland and Altman plots showing the mean *vs.* difference between the AWT parameters obtained from image analysis by one observer on 2 occasions are shown in Figure 4-5. In each case the mean difference was close to zero and there was no obvious relation between the measurement error and airway parameter. The scales used are the same as for the plots of inter observer variability.

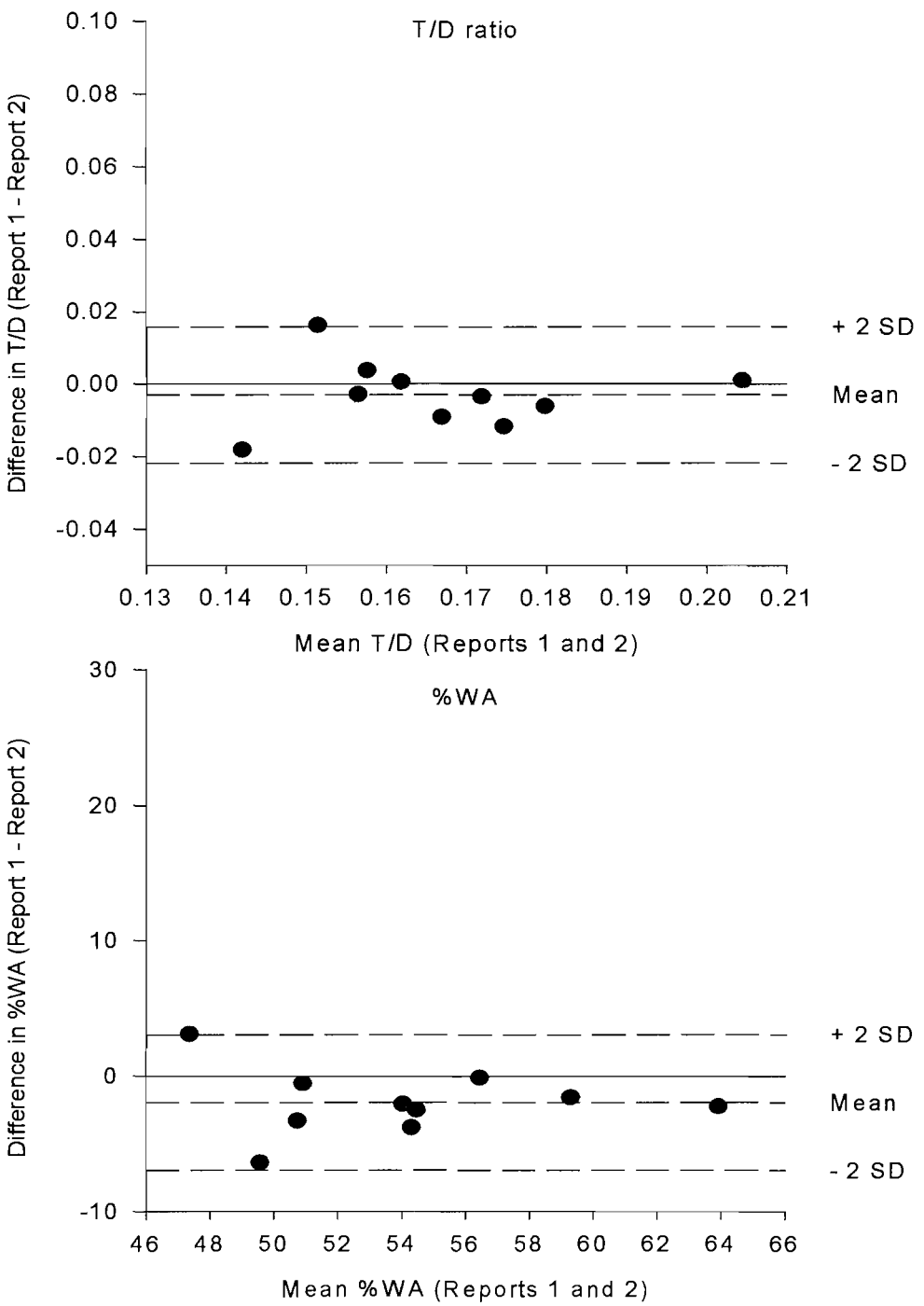


Figure 4-5 Bland-Altman plots showing intra observer agreement using EBUS

4.4.7 Within subject inter lobe variability of airway wall thickness using EBUS

Variation in AWT parameters between different sites in the lung are shown in Figure 4-6. The mean within subject inter lobe COV was 12% (T/D) and 10% (%WA).

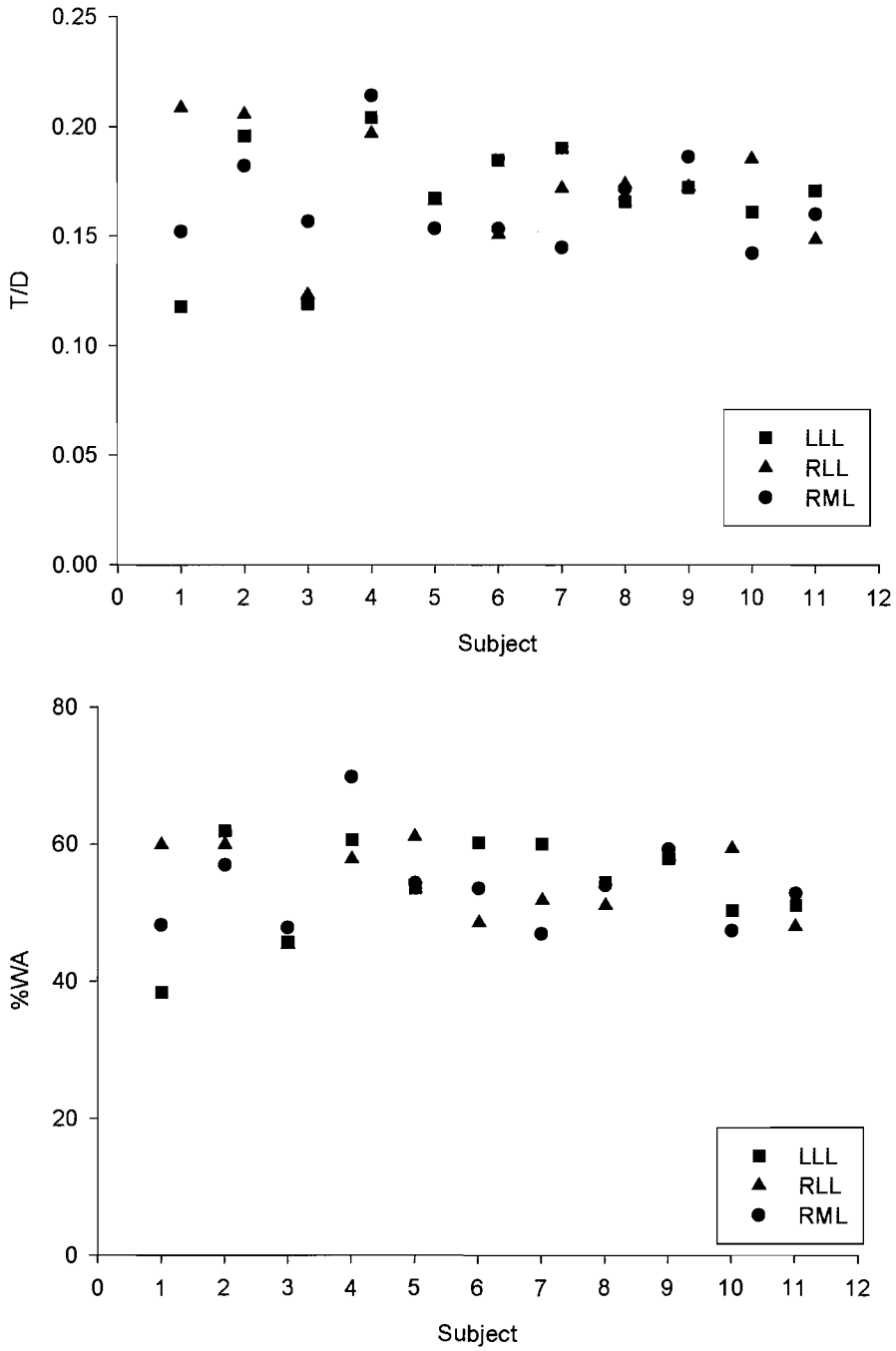


Figure 4-6 Inter lobe variability in AWT measurements using EBUS.

4.4.8 Mean within site repeatability of airway wall thickness using EBUS

The results of repeated measurements of AWT in asthmatics and controls taken at the same site are shown in Figure 4-7 for T/D and Figure 4-8 for %WA measures of AWT. The mean within site COVs for the 3 measurements of RLL AWT in the control group were 12% (T/D) and 8% (%WA). In the asthmatic group the COV was 11% (T/D) and 5 (%WA).

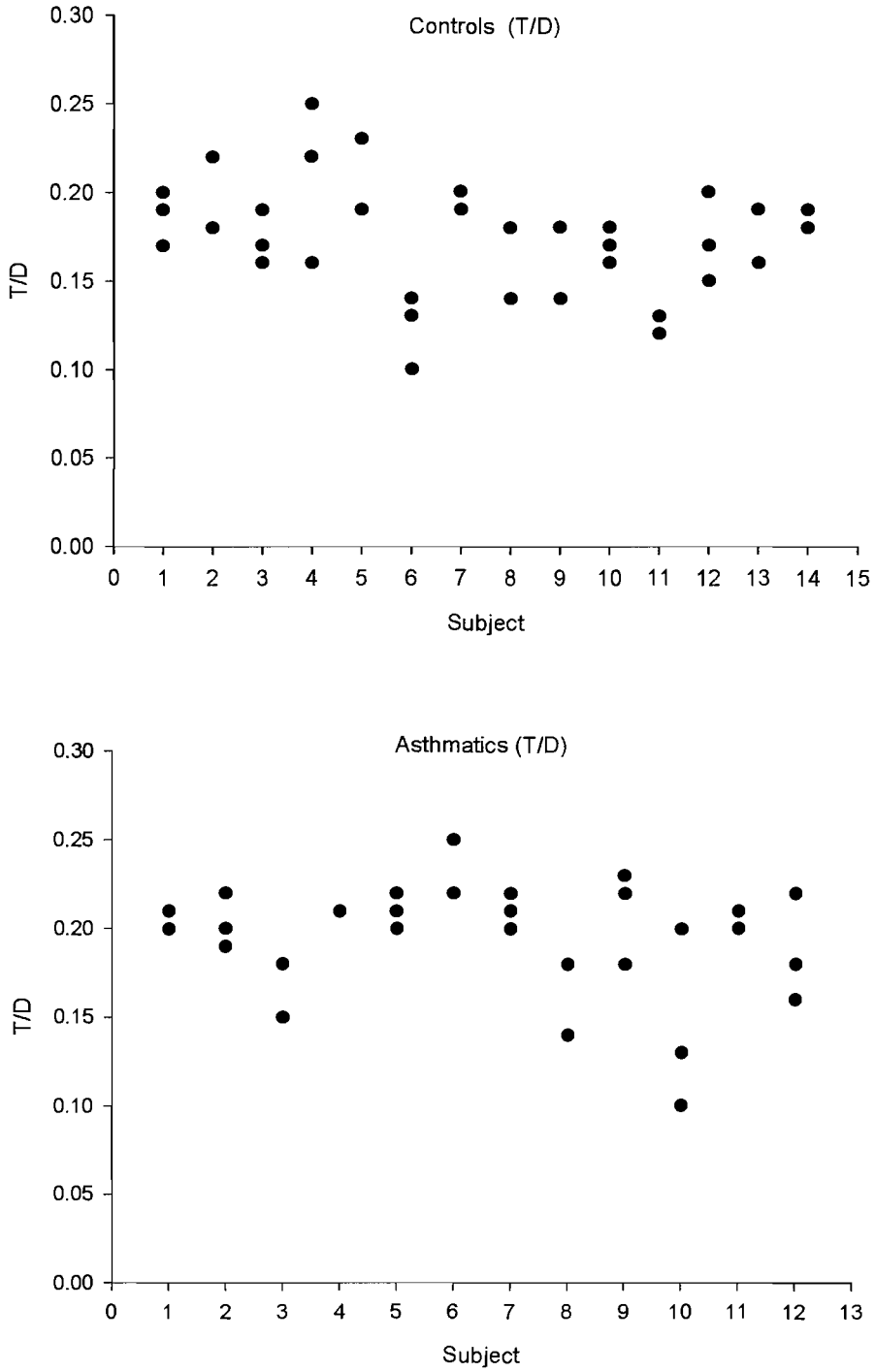


Figure 4-7 Same site repeatability of T/D measurements using EBUS

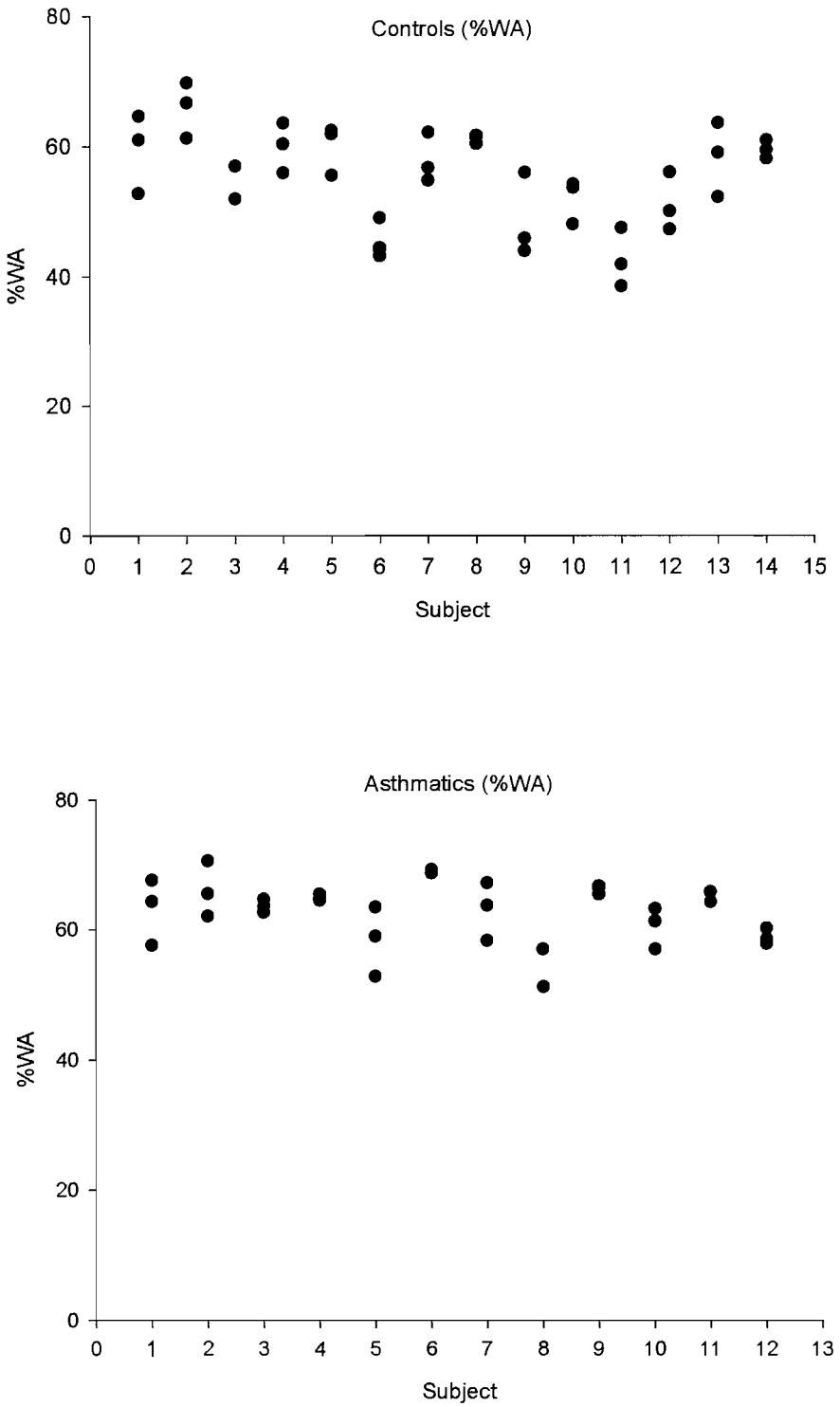


Figure 4-8 Same site repeatability of %WA measurements using EBUS

4.5 Discussion

4.5.1 Summary of results

A significant increase in total wall thickness was seen in asthmatics versus controls using both EBUS (T/D p = 0.002, %WA p < 0.001) and HRCT (T/D = 0.012, %WA p = 0.002), with no significant difference between the two methods (p < 0.001 for T/D and %WA)). Inter observer variability in EBUS measurements, assessed using the methods described by Bland and Altman showed a mean difference in T/D of less than 0.03 and %WA of less than 10%. Intra observer repeatability of EBUS measurements assessed in a similar way showed a greater level of agreement with a mean difference in T/D of less than 0.01 and %WA of less than 5%. Inter site coefficients of variation for measurements taken from 3 different lobes were found to be 12% (T/D) and 10% (%WA). Intra site coefficients of variation, to assess agreement between measurements taken from 3 different lobes, were found to be 12% (T/D) and 8% (%WA) for the control group and 11% (T/D) and 5% (%WA) in the asthmatic group. I conclude from these results that the increased AWT previously documented in asthmatics can be assessed using the technique of endobronchial ultrasound. Levels of inter and intra observer variability are within acceptable limits. Inter lobe variability is also within acceptable limits suggesting that measurement of AWT in one area of the lung can be taken as representative of the lung as a whole.

4.5.2 Methods for comparing wall thickness of airways of differing size

AWT decreases with airway diameter as one passes down the bronchial tree. Since all subjects have different sized airways, even in the same anatomical area, it is necessary to develop a method of relating wall thickness to airway size. Previous HRCT studies have employed various methods to achieve this, either relating wall thickness to airway calibre (135, 137, 138, 142) or comparing wall area or thickness to the total area within the airways external border. Wall/airway areas can be either measured directly at image analysis (136, 139-141) or derived mathematically from internal and external diameters using geometric formulae (138, 140, 142). One research group related wall area to body surface area (139, 141) (See Table 1-1 page 31 for summary of previous published HRCT work).

The majority of the post mortem studies of asthmatics have related airway wall area to, reticular basement membrane length, taken to represent the internal airway perimeter,

as this is believed to remain constant as varying lung volumes and degrees of smooth muscle shortening (85). Airways examined in this way show their mucosa thrown into folds whereas images obtained using HRCT or EBUS demonstrate a more regular luminal outline, presumably because airway surface liquid fills the spaces between the mucosal folds and *in vivo* airways are not in a collapsed state (136).

In this study I chose to use the parameters T/D and directly measured %WA since these involve the minimum mathematical manipulation of the data and have proved the most popular in the published literature. On repeating the analysis with wall area measurements derived from airway diameters, as favoured by some authors, I found no differences in the statistical outcomes.

4.5.3 Comparing airway wall thickness in asthmatics *vs.* controls

My results demonstrate airway thickening in asthmatics compared to controls, but not all studies using HRCT have found a similar increase in airway thickness in asthmatic subjects. Boulet *et al* studied 24 subjects with asthma (13 with fixed airway obstruction and 11 with variable obstruction and an FEV_1 >80%) and compared them with 10 controls (135). Significant thickening was only found in the asthmatic group with fixed obstruction. The authors suggested that their technique was not sensitive enough to pick up minor changes in stable asthmatics as they only examined a single central airway, the intermediate bronchus. Park *et al* studied 57 asthmatics and 10 controls and found the former to have thicker airway walls, but could not find any association with clinical features, lung function or bronchial hyperresponsiveness (137). In a small study of 6 mild to moderate asthmatics and 6 healthy volunteers Okazawa *et al* reported a greater wall area in asthmatics but this was confined to airways with an internal diameters of < 6mm and was not observed in larger airways (136). Interestingly they found that AWT in normal subjects decreased in response to methacholine but not in asthmatics and suggested that this was due to greater compliance and extrusion of blood and lymph fluids in the control group. Awadh *et al* examined 39 asthmatics (14 with near fatal asthma, 12 with moderate asthma, and 13 with mild asthma) and 14 controls (138). All asthmatic groups had thicker airways than controls, and the near fatal and moderate asthmatic groups had thicker airways than mild asthmatics or controls. The authors therefore concluded that airway thickness was related to asthma severity. In a large study using similar HRCT methods to examine the same central airway as I have, Niimi *et al* looked at 81 asthmatics (22 with severe persistent asthma, 39 with moderate asthma, 13 with mild asthma and 21

controls). They used measurements of absolute wall area (WA), % wall area (%WA) and the WA to body surface area ratio (WA/BSA), rather than wall thickness and found evidence of an increase in all measures in the severe and moderate, but not mild asthmatics, over controls. When all asthmatic subjects were analysed together all measures of wall area showed a weak but significant correlation with duration of disease and severity category. Only %WA correlated significantly with FEV_1. The authors hypothesised that there is a progressive increase in airway wall thickening with increasing duration as well as severity of asthma, the same conclusion as reached in a study of post mortem tissue (86). Kasahara *et al* studied 49 moderate asthmatics and 18 controls and found %WA and %wall thickness (%WT) to be significantly greater in the asthmatic group. They also took bronchoscopic biopsies and identified a significant correlation between both %WA and %WT with reticular basement membrane thickness and an inverse correlation with post bronchodilator FEV_1,suggesting remodelling has functional consequences (140). Gono *et al* recruited 24 asthmatics (14 with a degree of fixed and 10 with reversible airways obstruction) and 7 controls. HRCT measurement of AWT only demonstrated significant thickening in the group with fixed obstruction (179).

Despite almost universal agreement that airway wall thickening occurs in asthma and some common formulae for comparing airways of different diameter, there is variation between studies on the actual parameter values in asthmatics and controls. However my results are of a similar order to those previously published by other groups. Examples of a control and an asthmatic airway using HRCT are shown in Figure 4-9. A summary of my results and those from previously published work is given in Table 4-3.

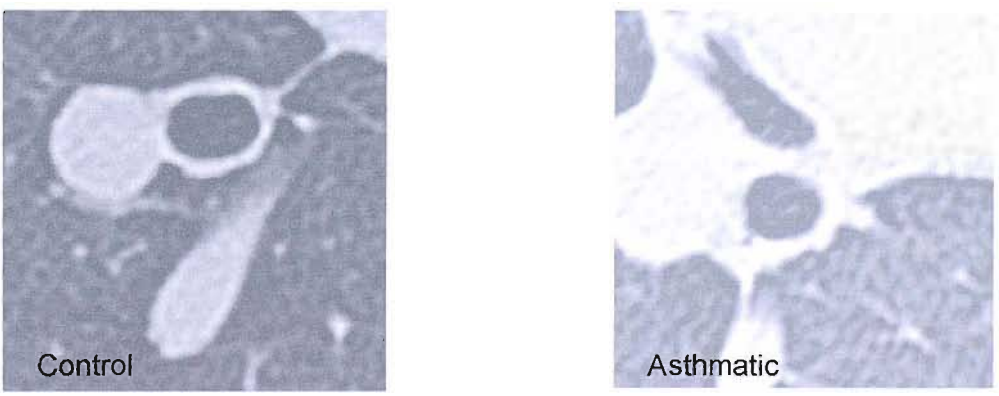


Figure 4-9 Examples of HRCT airway images from a control and asthmatic subject

Study	Protocol	T/D		%WA	
		Controls	Asthmatics	Controls	Asthmatics
My study using EBUS	Central airway	0.17	0.20	54	63
My study using HRCT	Central airway	0.16	0.19	49	58
Boulet *et al* HRCT (135)	Central airway	0.18	0.15 – 0.16	-	-
Okazawa *et al* HRCT (136)	5 levels φ 4-6mm	-	-	61	67
Awadh *et al* HRCT (138)	5 levels φ >2mm	0.21	0.22 – 0.24	67§	69 - 72§
Niimi *et al* HRCT (139)	Central airway	-	-	55	59 - 67
Little *et al* HRCT (142)	5 levels φ 2 - 10 mm	-	0.26§	-	76§
Kasahara *et al* HRCT (140)	5 levels φ 3 – 5mm	-	-	55	68
Gono *et al* HRCT (179)	Central airway	0.15	0.19	50	59

Table 4-3 Summary of HRCT AWT measurements from selected published work. Where results were broken down by airways size the diameter range closest to that I studied (φ 4-6mm) is shown. Figures shown are means for all subjects studied or ranges for asthmatics of different severities, where details are available. § Area calculated from measured diameters, - Result not reported

4.5.4 Image processing

There has been much debate about the most appropriate window/level settings for the interpretation of CT images (143, 180). The scale of Hounsfield units (HU) ascribed to different densities on CT scans is predetermined with 0 HU representing water density, -1000 HU air and +1024 dense bone. Window width/level settings of 1500/-450 to -600 HU have been accepted as most appropriate to provide the most accurate measurements of AWT (139, 143, 181). A similar scale does not exist for the interpretation of ultrasound images as contrast between hyper and hypoechoic tissue densities are more important than ascribed pixel values. The echo brightness of an ultrasound image changes continuously during recording, dependent on probe contact

and orientation, and is adjustable both on the machine in real time and on the saved DICOM images. This prevents standardisation of imaging even in a research setting. Operator experience in manipulation of the EBUS images to clarify the airway wall boundaries and direct measurements of intra and inter observer variability were an essential part of this study, and was found to be within acceptable limits.

4.5.5 Bronchus selection

In the first quantitative study to use HRCT to measure AWT in asthmatics Boulet *et al* selected the intermediate bronchus on the right side as it is usually cut perpendicularly to its axis in standard slices (135). Whilst this is convenient technically, the study of a single central airway raises the question of variation between sites in the lung. In a preliminary investigation to the study by Niimi *et al* the investigators examined the bronchus of the apical segment of the right upper lobe and the posterior basal bronchus of the right lower lobe, which is similarly in a favourable orientation. They found good correlation between airway measurements from the 2 locations ($p = 0.003$ for wall thickness and $p = 0.002$ for wall area) and only the first site was used in the full study to reduce radiation exposure (139). An HRCT based study of large and small airways found no significant difference in mean AWT in 5 slices obtained at different lung levels, and the authors suggest that wall thickening occurs uniformly throughout the lungs in asthma (140).

4.5.6 Inter and intra observer variability

The studies described so far in this section using HRCT or EBUS have required an operator to manually trace the margins of the airway by observation. Despite magnification and standardisation of viewing conditions a degree of operator variation is inevitable. Two HRCT based studies have examined inter and intra operator reproducibility using the methods of Bland and Altman described above. One found close limits of agreement and a mean difference close to zero in each case (139) but the other found group found a significant degree of inter observer variability (142). My results showed very good intra observer reproducibility with very close limits of agreement and a mean difference close to zero. Inter observer reproducibility was less good with limits of agreement of ± 0.04 for T/D (corresponding to 1% for a 4mm diameter airway) and ± 11% for %WA.

To avoid operator dependent variability attempts have been made to automatically measure airway dimensions from HRCT image data. It is possible to reconstruct airways in 3 dimensions and correct for orientation (144). Algorithms have been developed to automatically assess airway calibre in phantoms (dummy airways made of plastic or sweet potato) and animal models (144, 181, 182). This is relatively easy as a threshold level above air density allows identification of the airway wall. Automated assessment of AWT is more difficult to achieve as it requires identification of the external margin of the airway which is harder to define as it is often irregular due to adjacent soft tissue structures such as blood vessels and connective tissue parenchymal supports.

King *et al* developed an automated computed tomographic image analysis algorithm (CTAM) to measure airway lumen area (Ai), airway wall area (Awa) and airway angle of orientation in phantoms (plastic tubes embedded in Styrofoam) and fixed excised pig lungs (143). CTAM used an erosion method whereby, after manual identification of the centre of the airway, a large circle is whittled down on the basis of density scores for adjacent pixels until the outer margin of the airway is identified. Awa was calculated by subtraction of Ai assessed using a density threshold method. CTAM was compared with results from manual methods and was found to more accurately assess Ai, but both methods overestimated Awa in a diameter dependent manner. Nakano *et al* used an alternative method to study the right upper lobe apical segment bronchus in smokers with COPD and validated their method using phantoms (147). The airway lumen centre point was located and wall thickness (T) examined along 128 rays fanning out over 360º. The outer margin of the airway was taken as the point where the density fell to half its maximum along each ray. Rays were excluded it they were projected onto an adjacent vessel and T was calculated as the mean of all non excluded rays. The results on phantoms were within the range -2 to +4% for wall area and -11 to +9% for thickness. This protocol has also been used by Niimi *et al* to assessed asthmatic airways (141). Whilst the second protocol by Nakano gave more accurate results from phantoms, true validation *in vivo* was not addressed in either study. Measurement of a perfectly uniform phantom is very different to irregular airways and both protocols described above allowed user intervention to manually exclude adjacent vessels which can confound results.

4.5.7 Conclusions

These results have demonstrated that AWT is increased in asthmatics over control subjects. The results using EBUS are similar to those measured by HRCT methods and in broad agreement with similar studies in the published literature. Inter observer variability of EBUS image analysis is greater than intra observer variability, but both are within acceptable limits. Variability of EBUS measurements of AWT from different lobes is within acceptable limits and suggests that measurement in one area of the lung can be taken as representative of the lung as a whole.

Chapter 5: Airway wall thickening and bronchial hyperresponsiveness

5 Airway wall thickening and bronchial hyperresponsiveness

5.1 Introduction

One of the key aims of my research project was to investigate the relationship between AWT and bronchial hyperresponsiveness. This was based on the theory derived from mathematical modelling that modest degrees of airway wall thickening associated with only minimal changes in baseline resistance, will lead to enhanced bronchial hyperresponsiveness (72, 126, 183, 184).

5.2 Aims

1. To test the hypothesis that AWT in asthma is an important determinant of bronchial hyper-responsiveness.

2. To identify associations between AWT and other physiological measurements in asthmatics.

5.3 Methods

5.3.1 Study protocol

Asthmatic subjects were selected, recruited and physiologically characterised according to the methods described in chapter 2. Data from the first characterisation visit and the third bronchoscopy with EBUS visit by the asthmatic group were used in this part of the study (see Figure 2-1, page 42, for a summary of subject visits). Images obtained by EBUS were analysed according to the protocol previously described. For each subject 3 images were taken of the bronchus to the posterior segment of the right lower lobe. The mean AWT parameters, wall thickness to diameter ratio (T/D) and % wall area (%WA), measured by myself on the first occasion were used in subsequent analysis.

5.3.2 Statistical analysis

Subject characteristics and AWT parameters were summarised using the mean (SD) or median (range) as appropriate. Regression plots were constructed for the AWT parameters T/D and %WA *vs.* BHR, post bronchodilator FEV_1, β_2 reversibility and asthma duration. A Spearman Rank correlation coefficient was calculated in each case. A p value of < 0.05 was taken to indicate statistical significance.

5.4 Results

5.4.1 Subject characteristics

EBUS data on AWT was available for all 16 asthmatic subjects. Details of their baseline characteristics are shown in Table 5-1.

Subjects	Asthmatics
Number	16
Duration of asthma (years)	22 (11)
Age (years)	35 (13)
Inhaled steroids (11 subjects) (BDP mcg/day)	400 (0 – 2000)
FEV_1 (baseline, %predicted)	91 (13)
Reversibility to β_2 agonists (%)	13 (13)
PC_{20} (Histamine, mg/ml)	1.62 (0.07 – 8.00)

Table 5-1 Summary of subject characteristics

Data are mean (SD) or median (range)
BDP = Equivalent total daily dose of Beclomethasone Diproprionate

5.4.2 Airway wall thickness *vs.* bronchial hyperresponsiveness

Scatter plot with regression lines showing the relationship between BHR and AWT parameters, T/D and %WA, are shown in Figure 5-1. The Spearman Rank correlation coefficients were rho = 0.708 (p = 0.002) and rho = 0.560 (p = 0.024) for BHR *vs.* T/D and %WA respectively. Statistical analysis was carried out on the whole group in each case but steroid naïve and subjects taking inhaled corticosteroids are identified on the graphs.

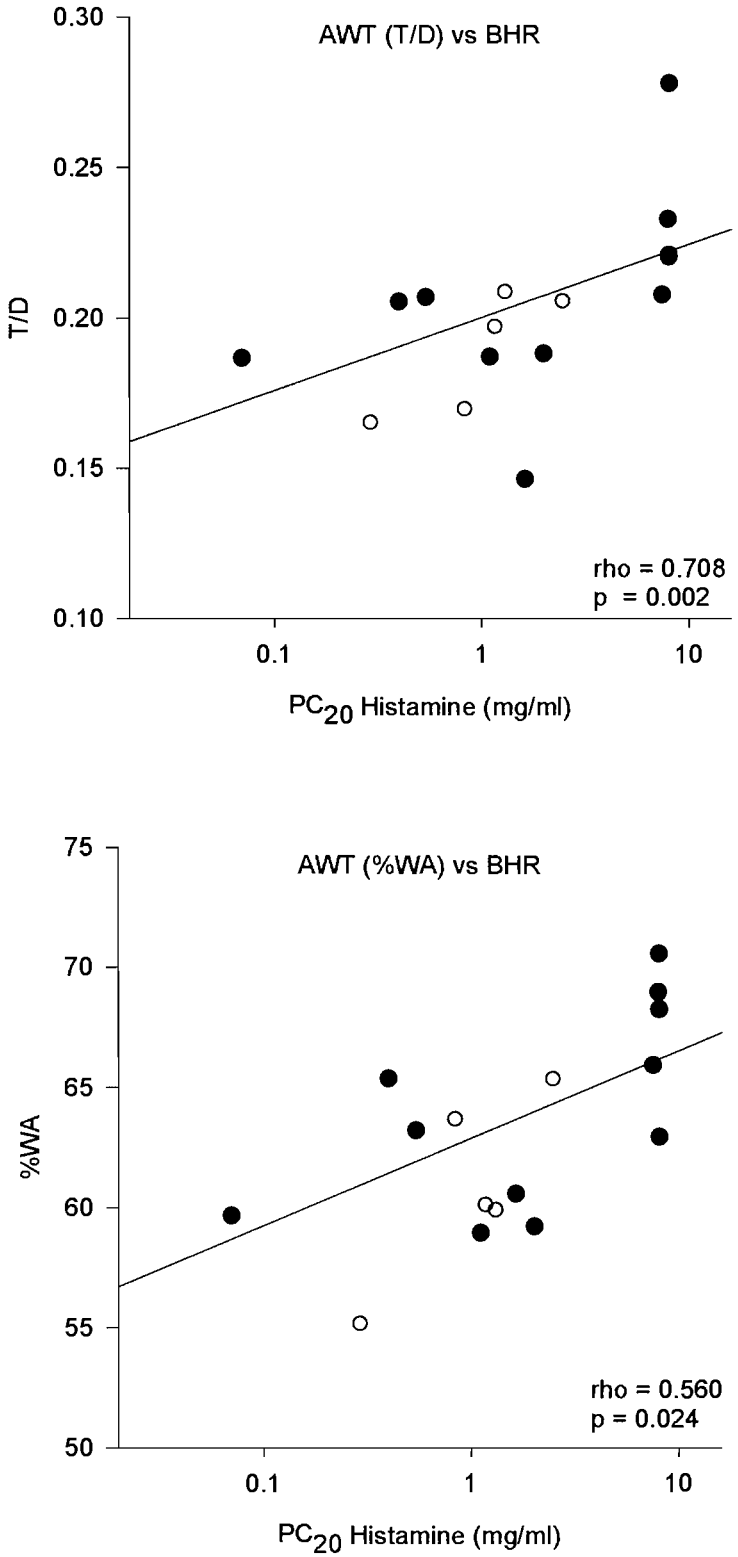


Figure 5-1 Regression analysis for BHR and AWT

○ steroid naïve subject ●subject taking inhaled corticosteroids.
Note the logarhythmic scale for BHR

5.4.3 Airway wall thickness and other pathophysiological parameters

The correlation between AWT and various pathophysiological parameters of the asthmatic subjects are shown in Figure 5-2, Figure 5-3 and Figure 5-4 below. A significant negative correlation was found between AWT (rho = -0.527, p = 0.036) and β_2 reversibility (rho = -0.503, p = 0.047). No significant association was seen between AWT and post bronchodilator FEV_1 and although a positive trend was seen between T/D and asthma duration, the reverse was seen with %WA.

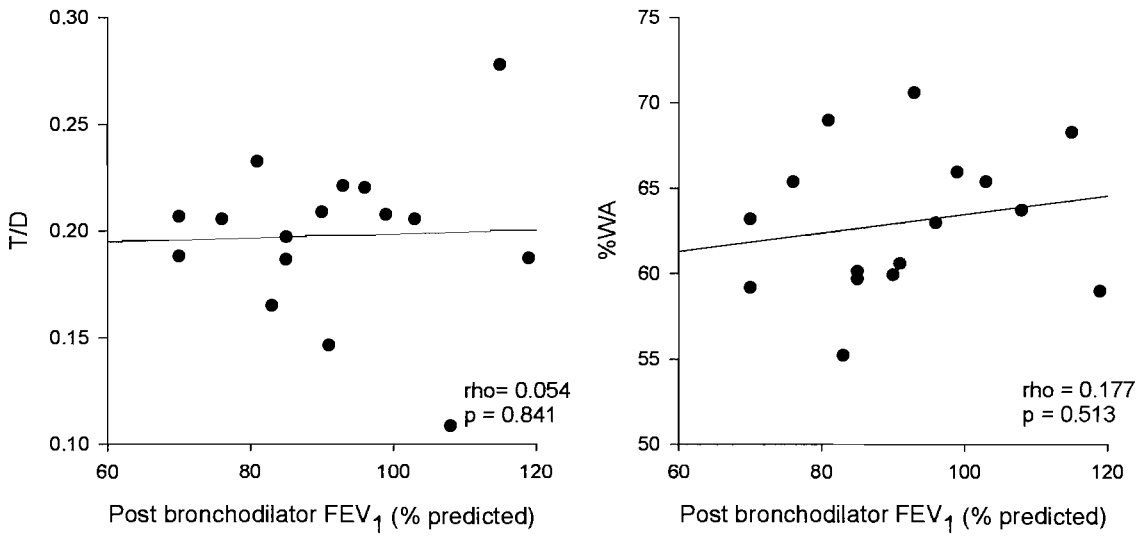


Figure 5-2 Regression analysis for post bronchodilator FEV_1 and AWT

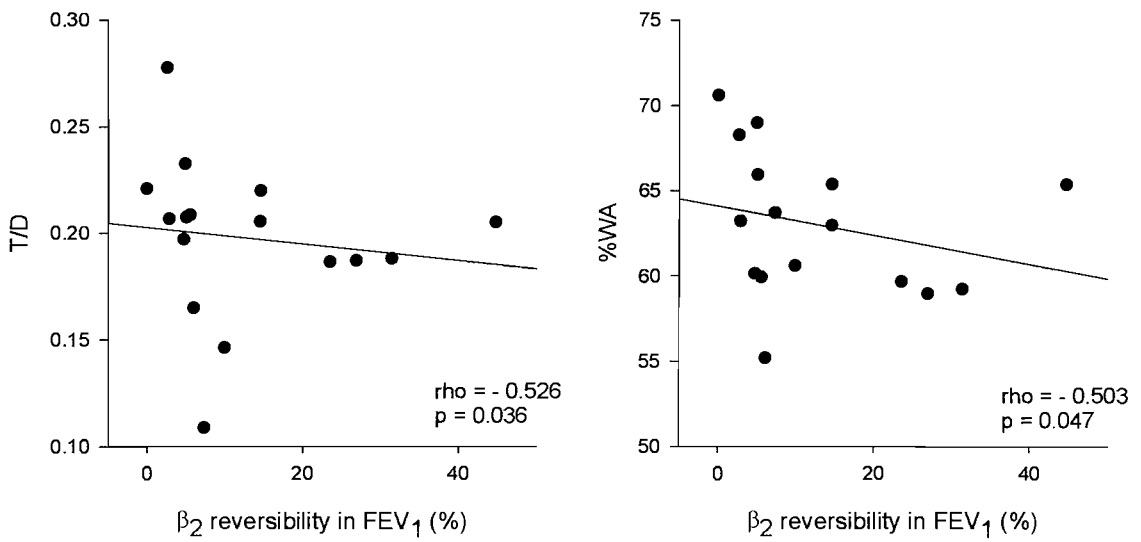


Figure 5-3 Regression analysis for β_2 reversibility and AWT

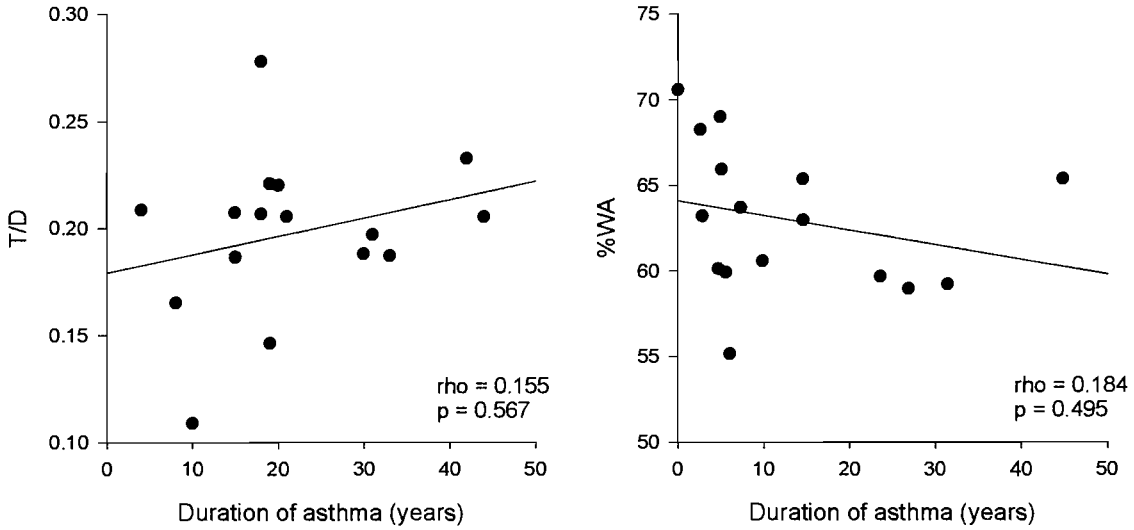


Figure 5-4 Regression analysis for asthma duration and AWT

5.5 Discussion

5.5.1 Summary of results

A significant positive correlation was found between AWT and PC_{20} (T/D rho = 0.708, p = 0.002, %WA rho = 0.560, p = 0.024), which equates to a negative relationship with bronchial hyperresponsiveness *i.e.* indicating airway wall thickening is associated with a lesser degree of airway reactivity. This is the opposite results to that predicted by the mathematical models, which propose enhanced reactivity from airway wall thickening. The use or otherwise of inhaled corticosteroids appeared to have no obvious effect on this relationship, though numbers in each group were too small to perform comparative statistical analysis. A significant negative correlation was found between AWT and reversibility to β_2 agonists (T/D rho = - 0.526, p = 0.036, %WA rho = - 0.503, p = 0.047). No significant association was found between AWT and post bronchodilator FEV_1 or asthma duration.

5.5.2 Airway thickness and bronchial hyperresponsiveness

Three published studies employing HRCT to study AWT in asthmatics *in vivo* have attempted to relate this to BHR. Boulet *et al* found a weak negative correlation between the T/D of the intermediate bronchus and PC_{20} to methacholine in a group of 13 asthmatics with fixed airflow obstruction (rho = - 0.65, p = 0.04) *i.e.* thicker airways were more responsive. No relationship was found in the group of 11 asthmatics with

variable airflow obstruction or 10 healthy controls (135). The authors suggested that their work supported the published theories on mathematical modelling of asthmatic airways, but the study was hampered by the small number of subjects studied and only one large central airway was examined which has a significant rigid cartilage component to its structure. In a similar study of 49 subjects whose asthma had been optimally controlled with corticosteroids Little *et al* failed to demonstrate a relationship between measures of AWT and BHR (142). This study looked at all airways large enough to be seen on 5 HRCT slices spread throughout the lungs, however it suffered from an acknowledged problem of interobserver variability. Both these studies used PC_{20} measurements to assess BHR, which is a marker of airway sensitivity and may also reflect reactivity as described in section 1.2.4. Niimi *et al* assessed both reactivity and sensitivity, by a continuous inhalation of methacholine method and identified a negative correlation between airway reactivity and AWT in both steroid naïve (n = 22, rho = - 0.56, p = 0.0049) and asthmatics taking inhaled steroids group (n = 23, rho = - 0.55, p = 0.007). No relationship was found between airway sensitivity and AWT in either group (141). Interestingly there was no relationship between airway sensitivity and reactivity in this study as previously described by some (33) but not all previous investigators (42, 43).

The effect of airway remodelling on the biomechanical properties of the airway wall and hyperresponsiveness will be discussed further in chapter 7.

5.5.3 Airway wall thickness and bronchodilator reversibility

I found a weak but significant negative correlation between both measures of AWT and reversibility to β_2 agonists i.e. wall thickening is associated with a reduction in reversibility to such medications. It is likely that the structural changes associated with airway remodelling in asthma, in particular the submucosal interstitial collagens, act as to oppose the actions of airway smooth muscle. Whilst this may lead to the detrimental effect of chronic airflow limitation and impaired reversibility to bronchodilators, it may also serve to protective the airway from excessive narrowing due to airway smooth muscle contraction. This theory will be discussed further in chapter 7.

5.5.4 Problems with mathematical modelling

The models proposed provide an elegant explanation for the way in which mucosal inflammation and remodelling, as causes of airway thickening, lead to increased BHR. There are however a number of weaknesses associated with the modelling studies.

- The morphometric data used to calculate changes in airway resistance are derived from studies of post mortem tissue from a very small number of patients, just 18 asthmatics and 23 controls (72).

- The architecture of the lung is not a symmetrical dichotomously branching tree and the airways do not act as a rigid set of pipes of circular cross section as assumed in the models. *In vivo* the branching structure is much more complex and airway diameters change dynamically during breathing, dependent on the opposing forces of airway pressure and smooth muscle contraction/elastic recoil.

- Relaxed airway diameters were derived from internal airway perimeters, with the assumption that basement membrane length remains constant at all lung volumes and degrees of smooth muscle contraction. This is necessary because at post-mortem airways are in a collapsed state and their diameter after fixation will depend on the inflation pressure used (85). This assumption has been challenged in a study where basement membrane length increased by about 50% when the transmural pressure was raised to 20 cm H_20 above zero. The authors argue that because at post mortem the airways of asthmatic patients, especially those dying of asthma, are constricted and not inflated with fixative, comparisons with normal subjects, where the lungs are not constricted leads to a systematic over estimate of airway wall compartment areas in asthma in studies employing this technique (185).

- The models are based on geometry alone and take no account of the interdependent biomechanical properties of the airway components. It is assumed that thickening of the airway smooth muscle (ASM) component will increase constrictive force and adventitial thickening will lead to uncoupling of the airway from the parenchyma and therefore reduce elastic recoil.

- An accepted weakness of the model is the assumption made about the percentage shortening of the smooth muscle derived from animal experiments (184). A single *in vivo* study of the trachealis muscle in the dog is cited, where 28% shortening was seen. Several more studies have examined ASM shortening of explanted material

in vitro, with levels up to 70% of the resting length reported (84, 186, 187). In the published study by Wiggs *et al* a figure of 30-40% shortening was arbitrarily chosen, which may be too generous (183).

The possible protective effect of airway remodelling in reducing excessive airway constriction will be discussed more fully in the general discussion chapter 7.

5.5.5 Airway wall thickness and inflammation

Neither HRCT nor EBUS are able at present to identify different component structures within the airway wall. An increase in AWT observed in asthmatics could be due to an increase in structural tissues such as collagens and smooth muscle as part of the remodelling process, or due to oedema associated with chronic inflammation.

The interrelationship between inflammation and remodelling is complex with some researchers favouring the view that chronic inflammation leads to remodelling (188) whilst others believe the processes occur in parallel (189). Depletion of bronchial eosinophils *in vivo* (with infusions of an anti-IL 5 monoclonal antibody) has been shown to decrease TGF-β expression and reverse remodelling, as measured by deposition of tenascin, lumican and procollagen III in the RbM (190). Similar attenuation of the remodelling response to allergen challenge has been demonstrated in a transgenic mouse model unable to generate eosinophils, though BHR and mucous production was unaffected (191). Transgenic mice deficient in IL 5 have also shown reduced airway remodelling and TGF-β production (192).

Airway oedema has been shown to increase HRCT measurements of AWT in anaesthetised dogs treated with large volumes of intravenous fluids, where it was associated with an increase in bronchoconstriction to inhaled histamine (193).

Airway Inflammation in asthmatics is variable over time dependent on allergenic or infective exposures and anti inflammatory medication. Chronic inflammatory changes can be demonstrated using BAL or bronchial biopsy techniques (52) and have been shown to occur in mild (78) and newly diagnosed asthmatic subjects (67). Treatment with oral or inhaled corticosteroids has been shown to reduce inflammatory cell numbers and improve bronchial hyperresponsiveness and symptoms (58, 59, 65, 194).

Some HRCT based studies have specifically tried to minimise any reversible inflammatory component to airway thickening by treatment with oral or high dose inhaled corticosteroids for a number of weeks prior to imaging (140, 142). Whilst initially this idea is attractive only 2 or 4 weeks pre-treatment was given in these studies which will suppress inflammation to a variable degree but is unlikely to eliminate it entirely. A study by Ward *et al* of mild but symptomatic asthmatics found the effects of high dose inhaled corticosteroids on airway inflammation, lung function and bronchial hyperresponsiveness were not temporally concordant (195). Inhaled fluticasone propionate, 750mcg twice daily, improved FEV_1 and BAL inflammatory cell counts by 3 months with no further improvement at 12 months. Bronchial hyperresponsiveness improved more slowly and throughout the 12 month study period. Prolonged treatment also reduced basement membrane thickness at 12 months suggesting an effect on remodelling and therefore potentially the structural element of airway thickening. I elected to select subjects who were clinically stable and maintained them on their usual steroid medication throughout bronchial hyperresponsiveness and airway thickness assessments. In doing so I accept that inflammation contributes in part to the increase in AWT seen in the asthmatic subjects, but this should correspond more accurately with the degree of bronchial hyperresponsiveness measured without the confounding effect of a change in therapy on the parameters being measured.

5.5.6 Airway wall thickness and bronchoconstriction

Bronchoconstriction has been shown to decrease bronchial wall thickness in dogs (144) and human control subjects but not asthmatics (136). The authors of these studies speculated that this was due to a reduction in bronchial wall blood volume. Prior to bronchoscopy and EBUS measurements of AWT both asthmatic and control subjects received a nebulised β_2 agonist to permit maximal bronchodilatation and reduce any effects of bronchoconstriction on AWT.

5.5.7 Airway wall thickness and clinical indices of asthma severity

A number of the studies using HRCT to assess AWT have attempted to correlate their findings with clinical indices of asthma severity. Awah *et al* subdivided asthmatics according to ATS criteria (196) into near fatal asthma (previous episode of hypercapnoea or requirement for mechanical ventilation), moderate asthma (requiring > 1000mcg inhaled steroids/day) and mild asthma (FEV1 > 80% and < 1000mcg inhaled steroids/day). AWT was greater in the near fatal/moderate groups than the

mild asthma group and the authors suggested AWT was related to disease severity (138). Other studies have also described associations between asthma severity based on ATS (196), Aas (197) and GINA (3) severity scores (139, 142). These scoring systems are partly subjective, particularly if subjects have been started on treatment and severity has to be assigned retrospectively.

Early studies by Boulet *et al*, Park *et al* and later Little *et al* reported no significant association between AWT and baseline FEV_1, FEF_{25-75} and sGaw (135, 137, 142). More recent studies by Niimi, Kasahara, and Gono have shown an inverse relationship between various measures of AWT and either baseline or post bronchodilator FEV_1 (139, 140, 179). In my study I found no such a relationship, possibly because many of my subjects had only mildly impaired lung function with an FEV_1 >80%. At a physiological level a simple association is not expected as the effect of increased AWT on airway calibre is minimal and its effects on airway mechanics complex.

5.5.8 Conclusions

AWT is inversely correlated with bronchial hyperresponsiveness, which is in contrast to that proposed by mathematical modelling. AWT is negatively correlated with reversibility to β_2 agonists. No significant association was found between AWT and post bronchodilator FEV_1 or asthma duration.

Chapter 6: Airway wall thickening and mucosal markers of remodelling

6 Airway wall thickening and mucosal markers of remodelling

6.1 Introduction

Over the last 20 years there has been much interest in the pathological changes associated with asthma. Many studies have employed bronchoscopic biopsy and lavage techniques to describe the inflammatory changes in asthmatic subjects (51-56). Subsequent intervention studies have shown that treatment with inhaled or oral corticosteroids can significantly, though almost certainly incompletely, reduce this inflammation, and lead to clinical improvement in symptoms (56, 58, 59, 65, 195, 198-203). Bronchial biopsy studies have also been able to demonstrate the features of airway remodelling in asthmatics and there is some evidence that these too may at least partially be reversed by corticosteroids (194, 195, 204-209). There is little published data comparing whole airway wall thickening with traditional markers of remodelling, such RbM thickening and extracellular matrix deposition of collagens and proteoglycans.

6.2 Aims

To confirm the expected presence of airway remodelling in bronchoscopic biopsies taken from asthmatic subjects compared with non asthmatic controls.

To examine the hypotheses that increases in whole wall thickening correlate with airway remodelling as assessed by RbM thickness, interstitial collagen and proteoglycan deposition.

To examine the hypothesis that bronchial hyper responsiveness is associated with increased airway remodelling as assessed by RbM thickness, interstitial collagen and proteoglycan deposition.

6.3 Methods

6.3.1 Study protocol

Full details of the methods used are given in chapter 2. Briefly, asthmatic and control subjects were physiologically characterised before undergoing fibreoptic bronchoscopy with EBUS measurements of AWT in a sub-segmental airway of the right lower lobe. Three endobronchial biopsies were taken from the same site at the same time. These biopsies were fixed and processed into GMA resin before staining using mono or polyclonal antibodies to identify eosinophils and the structural components of remodelling (collagens I, III and V, proteoglycans tenascin, decorin, fibronectin, biglycan and perlican and smooth muscle actin). See Table 2-1 and Table 2-2 (page 53) for details of the primary and secondary antibodies used. Labelled structures were visualised using a chromogen and slides were counterstained using haematoxylin to show the background architecture of the biopsies. RbM thickness was assessed by image analysis of biopsies stained with Toluidine Blue. Submucosal eosinophils were counted manually and expressed as a number per square millimetre of submucosal tissue. αSMA staining was assessed and expressed as a percentage of the whole biopsy area. Interstitial collagen and proteoglycan staining of the submucosa was assessed using image analysis software. Sub mucosal tissue excluding the RbM, blood vessels and smooth muscle was analysed with results being expressed as % staining of the submucosa. In addition to the control and mild/moderate asthmatics studied in previous chapters and for whom AWT measurements were available, a third group of 9 severe asthmatics were also studied. These came from historical bronchial biopsies taken from severe asthmatics in this department in the past and not by myself. Subjects were physiologically characterised in a similar manner but many were not able to undertake assessment of their bronchial hyperresponsiveness due to poor baseline spirometry. Data on reversibility to β_2 agonists was also not available. Details of the subject characteristics are given below.

6.3.2 Statistical analysis

Subject characteristics and AWT parameters were summarised using the mean (SD) or median (range) as appropriate. Box plots with markers of 10^{th}, 25^{th}, 50^{th}, 75^{th} and 90^{th} percentiles were constructed with all outliners being shown. The Mann Whitney U test was used to assess differences between subject groups. Regression plots were constructed for the AWT parameters T/D and %WA and for BHR verses markers of

airway remodelling. A Spearman Rank correlation coefficient was calculated in each case. A p value of < 0.05 was taken to indicate statistical significance.

6.4 Results

Biopsy data was available for all 15 control and 12 of the mild/moderate asthmatic subjects. Details of their baseline characteristics and those of the severe asthmatics that did not undergo EBUS measurements of AWT are shown in Table 6-1.

Subjects	Controls	Asthmatics mild/moderate	Asthmatics severe
Number	15	12	9
Duration of asthma (years)	-	22 (13)	16 (11)
Age (years)	40 (3)	37 (14)	48 (13)
Inhaled steroids (BDP mcg/day)	-	300 (0 – 2000)	2000 (1600 – 4000)
Oral prednisolone (mg/day)	-	-	25 (0 – 40)
FEV_1 (baseline, %predicted)	106 (3)	90 (13)	61 (18)
Reversibility to β_2 agonists (%)	0 (5)	12 (12)	-
PC_{20} (Histamine, mg/ml)	>8	1.1 (0.1 – 8.00)	-

Table 6-1 Subject baseline characteristics

Data are mean (SD) or median (range)
BDP = Equivalent total daily dose of Beclomethasone Diproprionate

6.4.1 Sub mucosal eosinophils

Results were available for biopsies from the mild/moderate asthmatic and control groups only. The density of eosinophils in mucosal biopsies was greater in asthmatic subjects than in controls, but this difference did not reach significance, as shown in Figure 6-1. Figure 6-2 shows an example of an eosinophil from one of the slides from an asthmatic subject stained for the cell surface marker on activated eosinophils EG2.

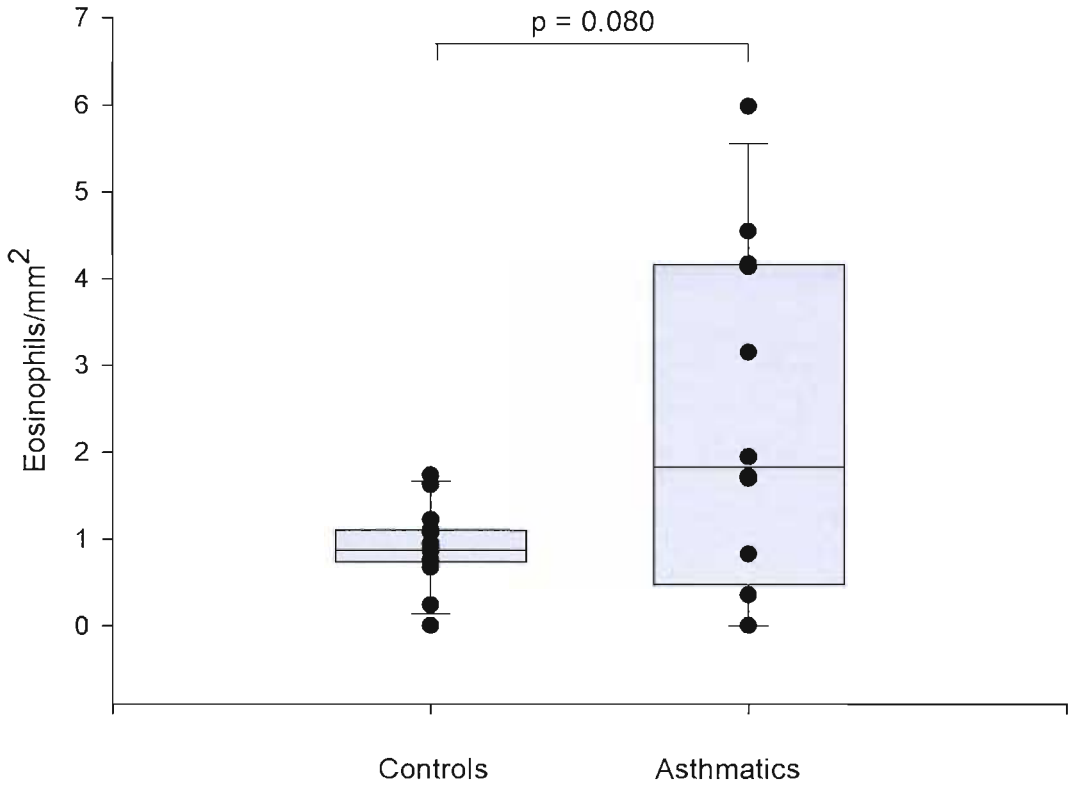


Figure 6-1 Submucosal eosinophils in mild/moderate asthmatics and control subjects

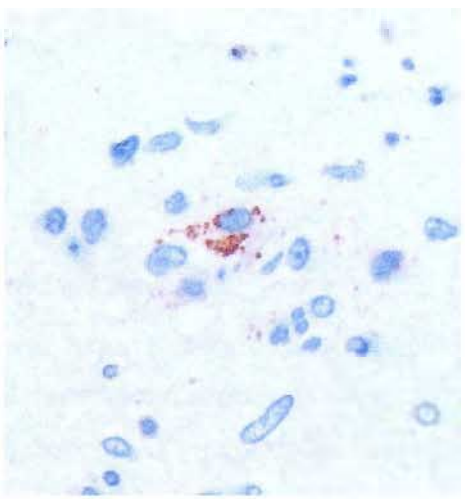


Figure 6-2 Example of an eosinophil stained for EG2

EG2 positive granules within the eosinophils are visualised by red staining

When the data was broken down by medication usage in the mild/moderate asthmatic group, only the steroid naïve (using β_2 agonists only) group of asthmatics showed significantly greater numbers of eosinophils than controls, as shown in Figure 6-3.

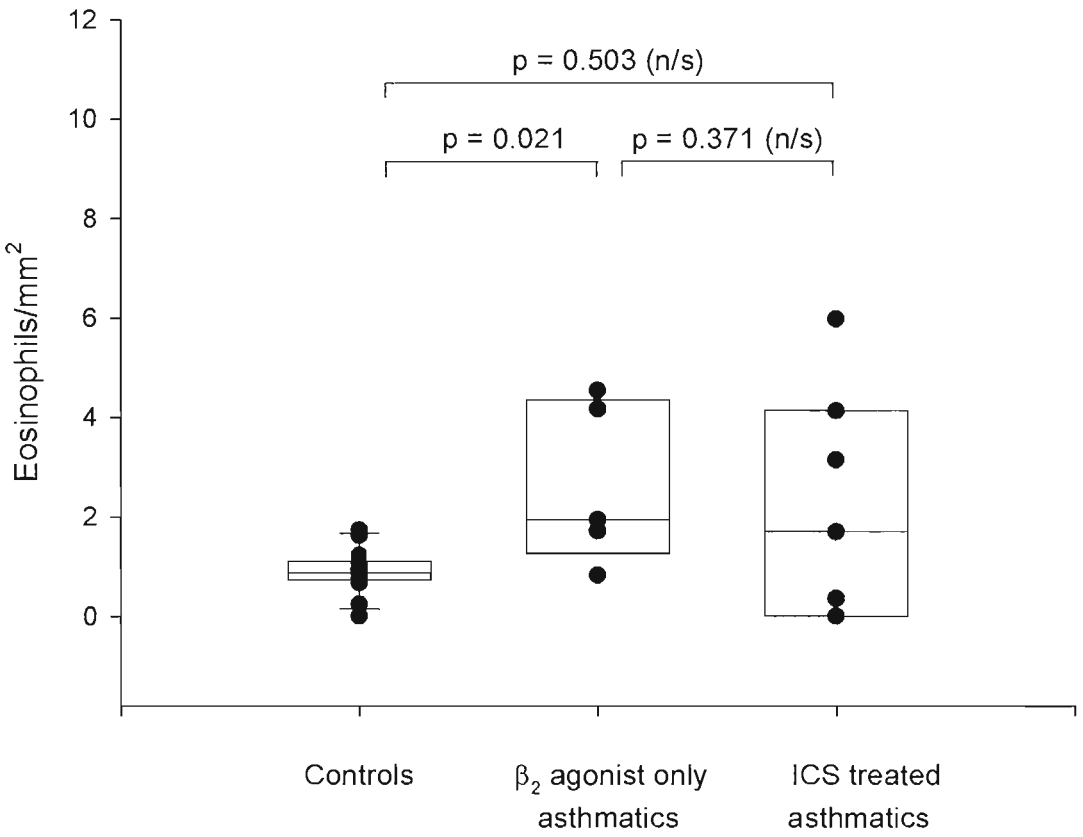


Figure 6-3 Submucosal eosinophils in mild/moderate asthmatics and controls subdivided by medication
ICS = inhaled corticosteroid

6.4.2 Reticular basement membrane thickness

The thickness of the RbM was found to be increased in asthmatic subjects over controls, see Figure 6-4. Corticosteroid use made no apparent difference to the results, see Figure 6-5. An example of an endobronchial biopsy stained with toluidine blue to demonstrate the RbM is shown in Figure 6-6.

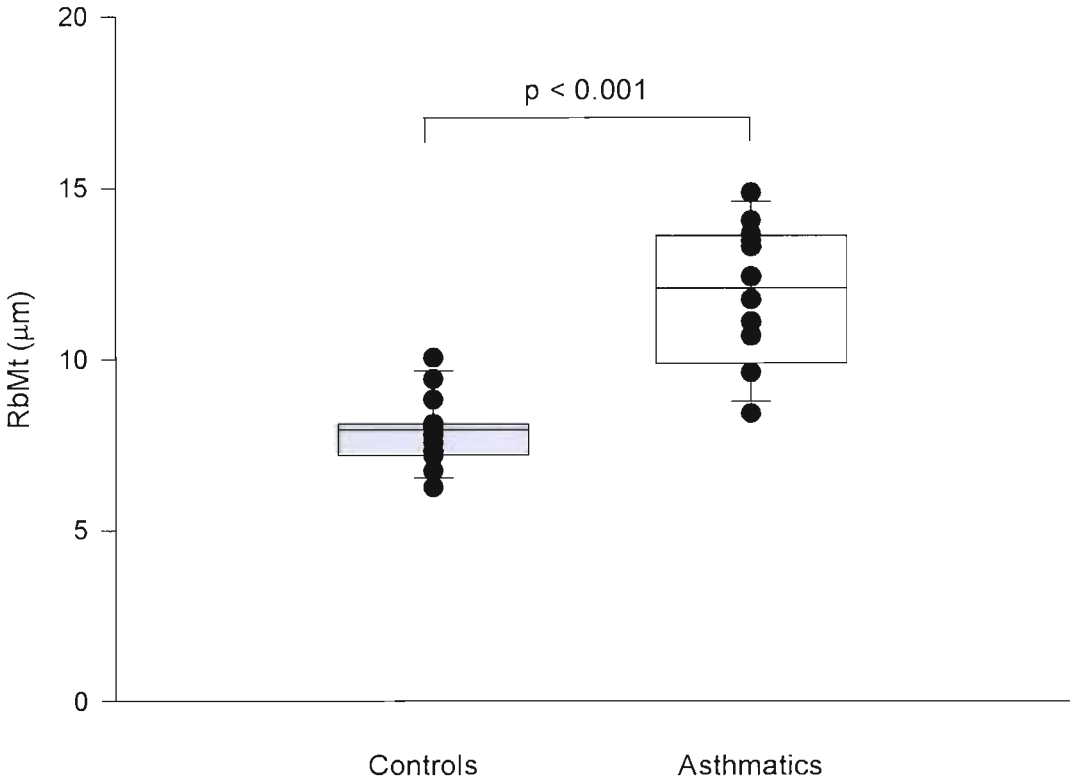


Figure 6-4 RbM thickness in mild/moderate asthmatic and control subjects

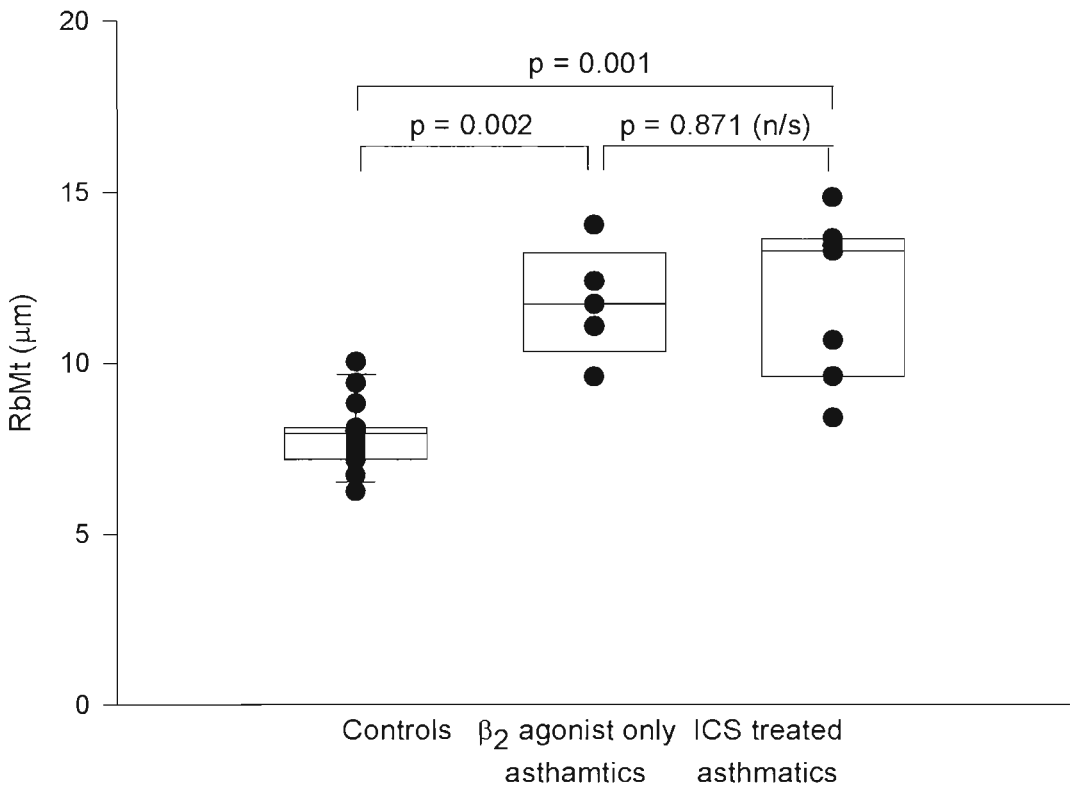


Figure 6-5 RbM thickness in asthmatic and control subjects, subdivided by medication
ICS = inhaled corticosteroid

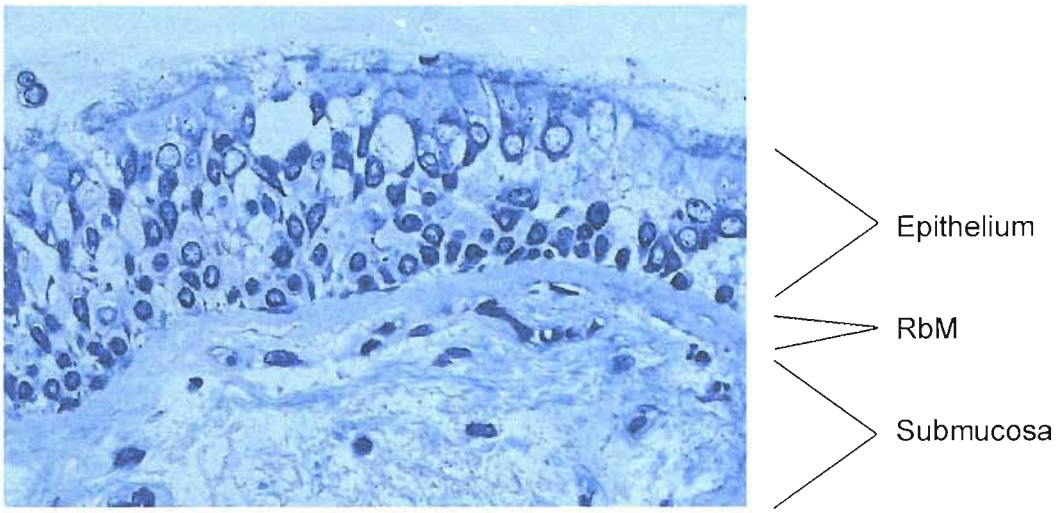


Figure 6-6 Example of endobronchial biopsy stained with toluidene blue to show RbM (cut tangentially)

6.4.3 Sub mucosal αSMA

The fraction of airway biopsy section area stained by antibodies to αSMA did not differ between asthmatic and control subjects. See Figure 6-7.

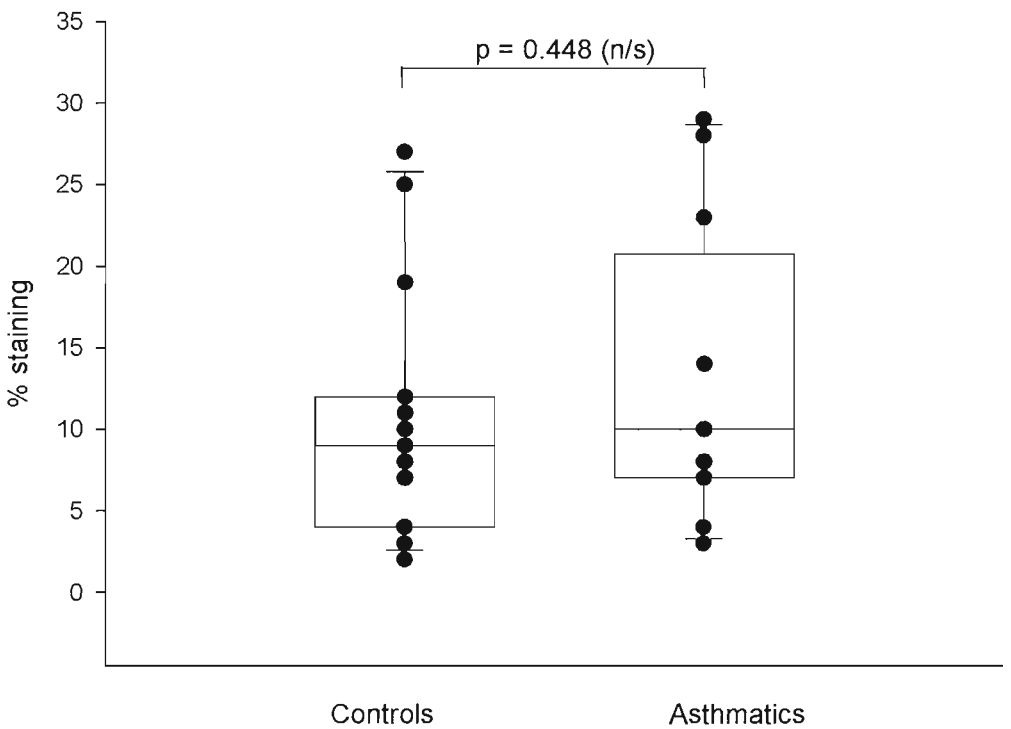


Figure 6-7 αSMA in mild/moderate asthmatics and control subjects

6.4.4 Sub mucosal interstitial collagens

Interstitial collagens I, III and V were increased in asthmatic subjects when compared to controls. Staining for collagens III and V showed an increasing trend with disease severity as shown in Figure 6-8, Figure 6-10 and Figure 6-11. Examples of endobronchial biopsies stained for collagen I from an asthmatic and control subject are shown in Figure 6-9.

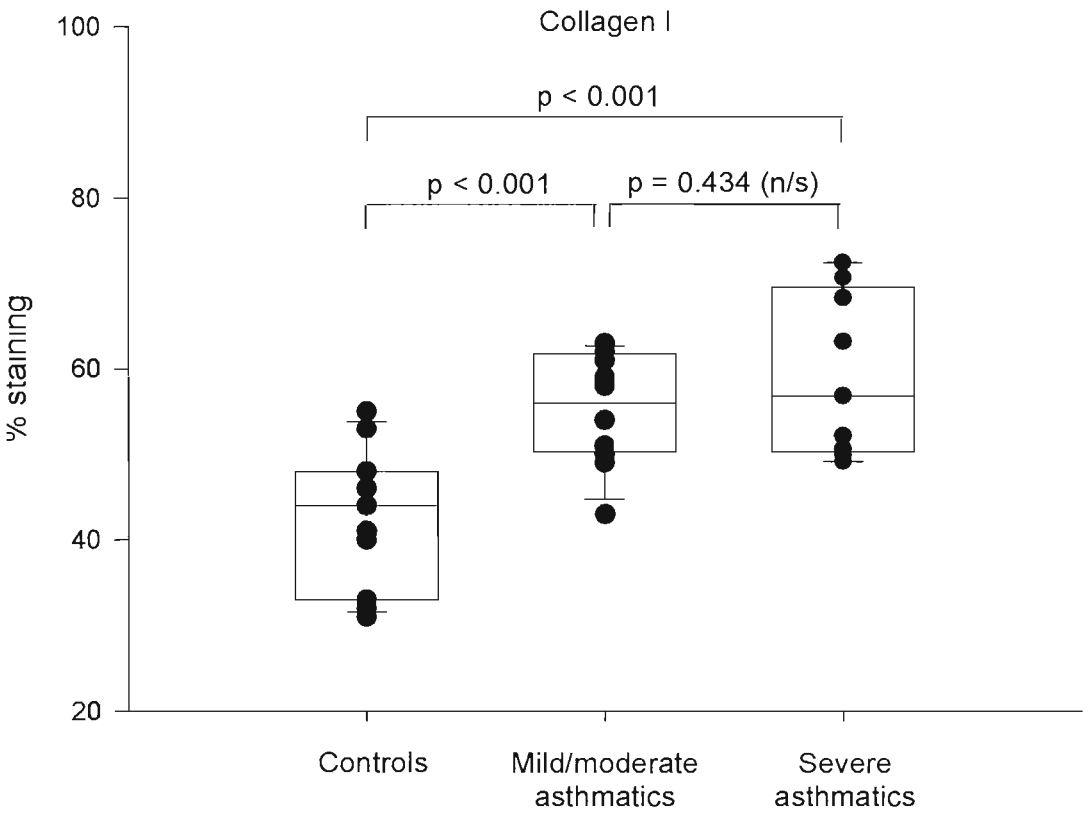


Figure 6-8 Collagen I in mild/moderate and severe asthmatics and controls

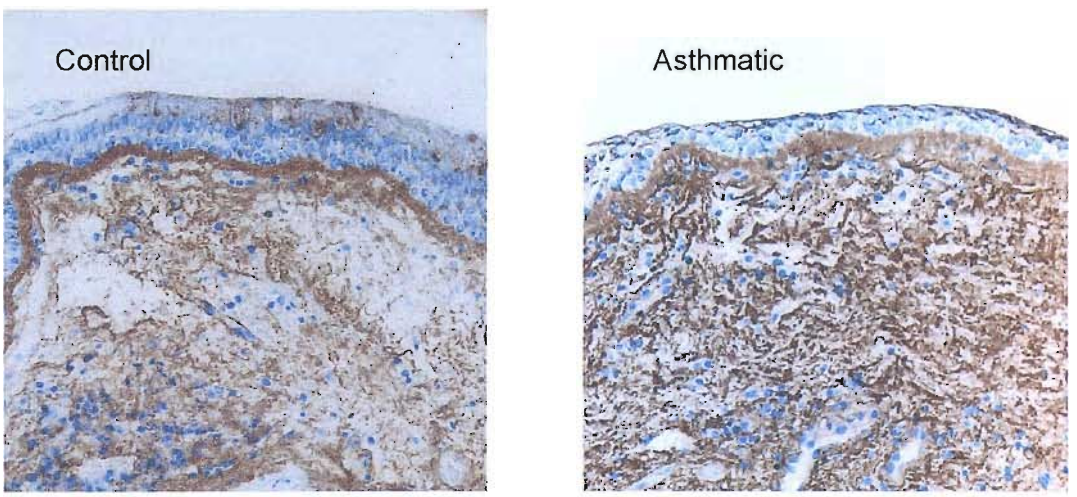


Figure 6-9 Examples of endobronchial biopsies stained for collagen I

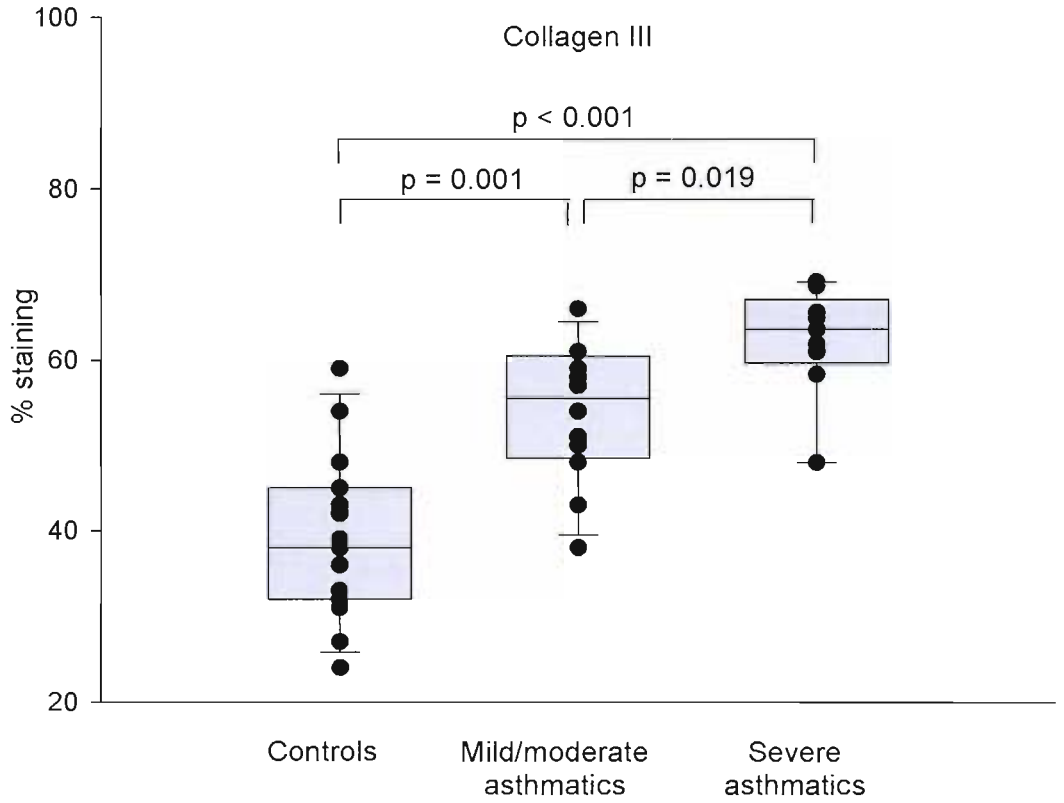


Figure 6-10 Collagen III in mild/moderate and severe asthmatics and controls

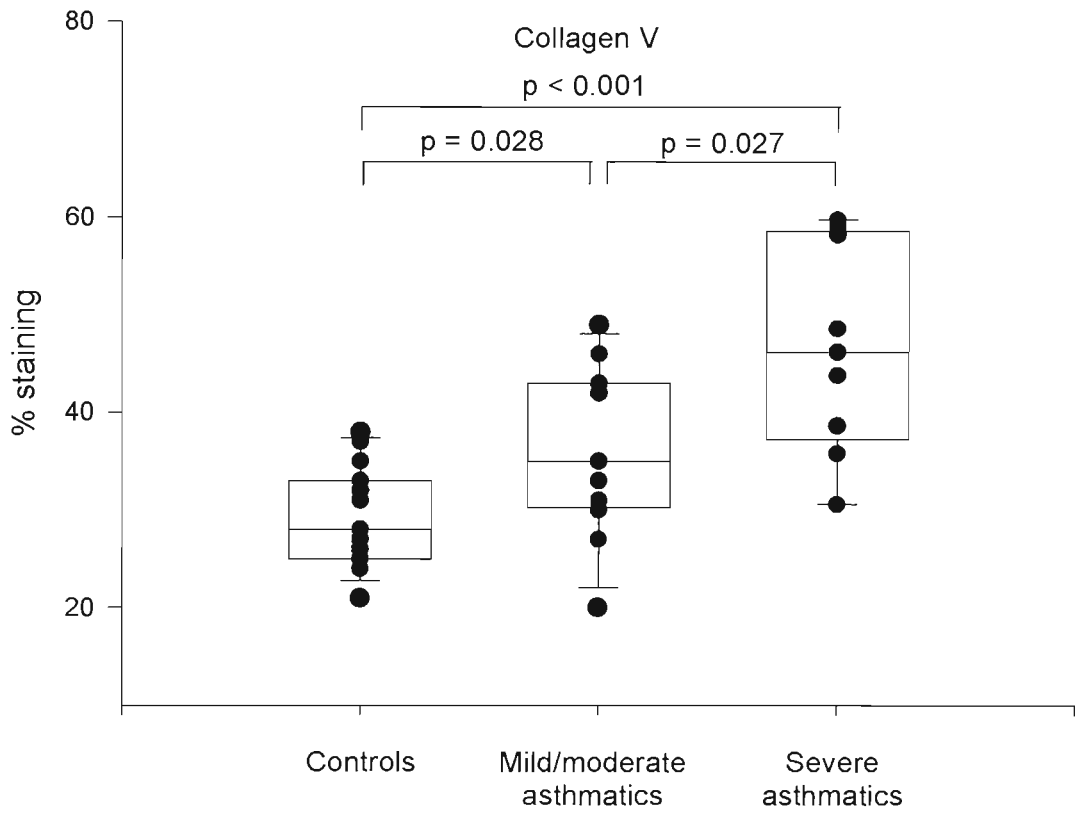


Figure 6-11 Collagen V in mild/moderate and severe asthmatics and controls

6.4.5 Sub mucosal proteoglycans

The sub mucosal staining for the proteoglycans perlican, biglycan, decorin and fibronectin, was increased in asthmatic subjects compared to controls. In the case of perlican this was found to be associated with disease severity. Staining for tenascin was not found to be different between the two groups. See Figure 6-12 and Figure 6-13. An example of an endobronchial biopsy stained for tenescin from an asthmatic subject is shown in Figure 6-14.

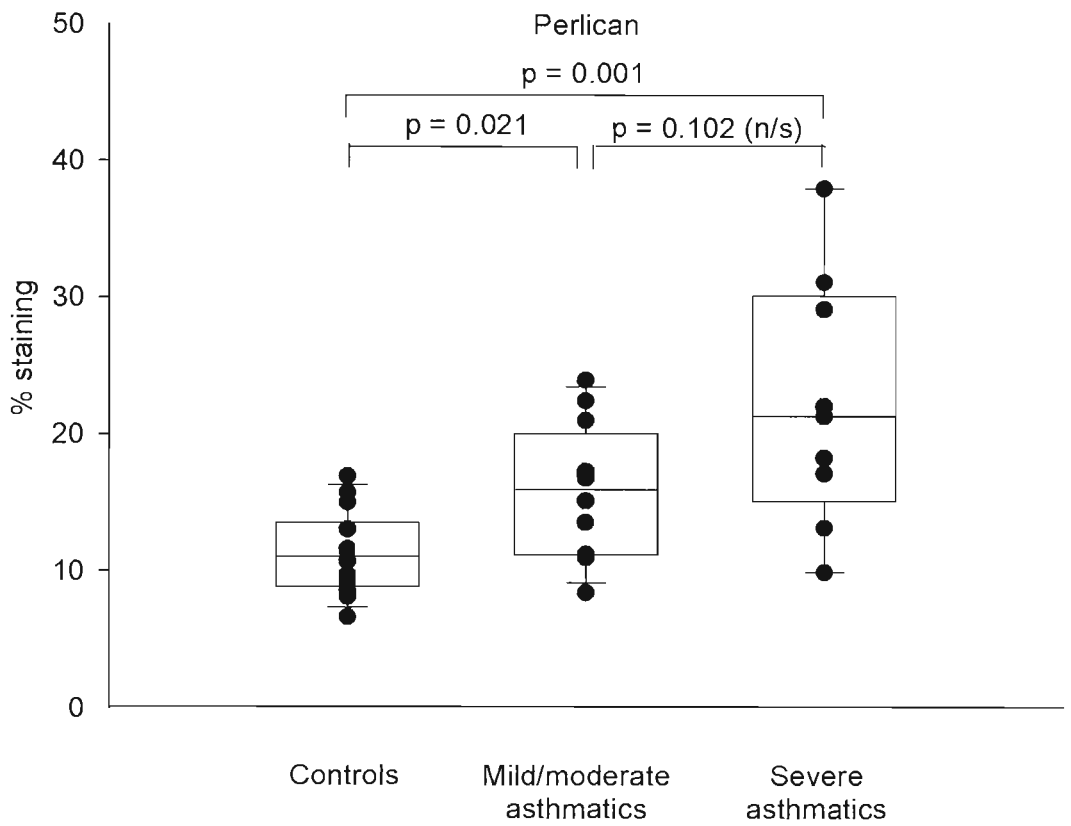


Figure 6-12 Perlican in mild/moderate and severe asthmatics and controls

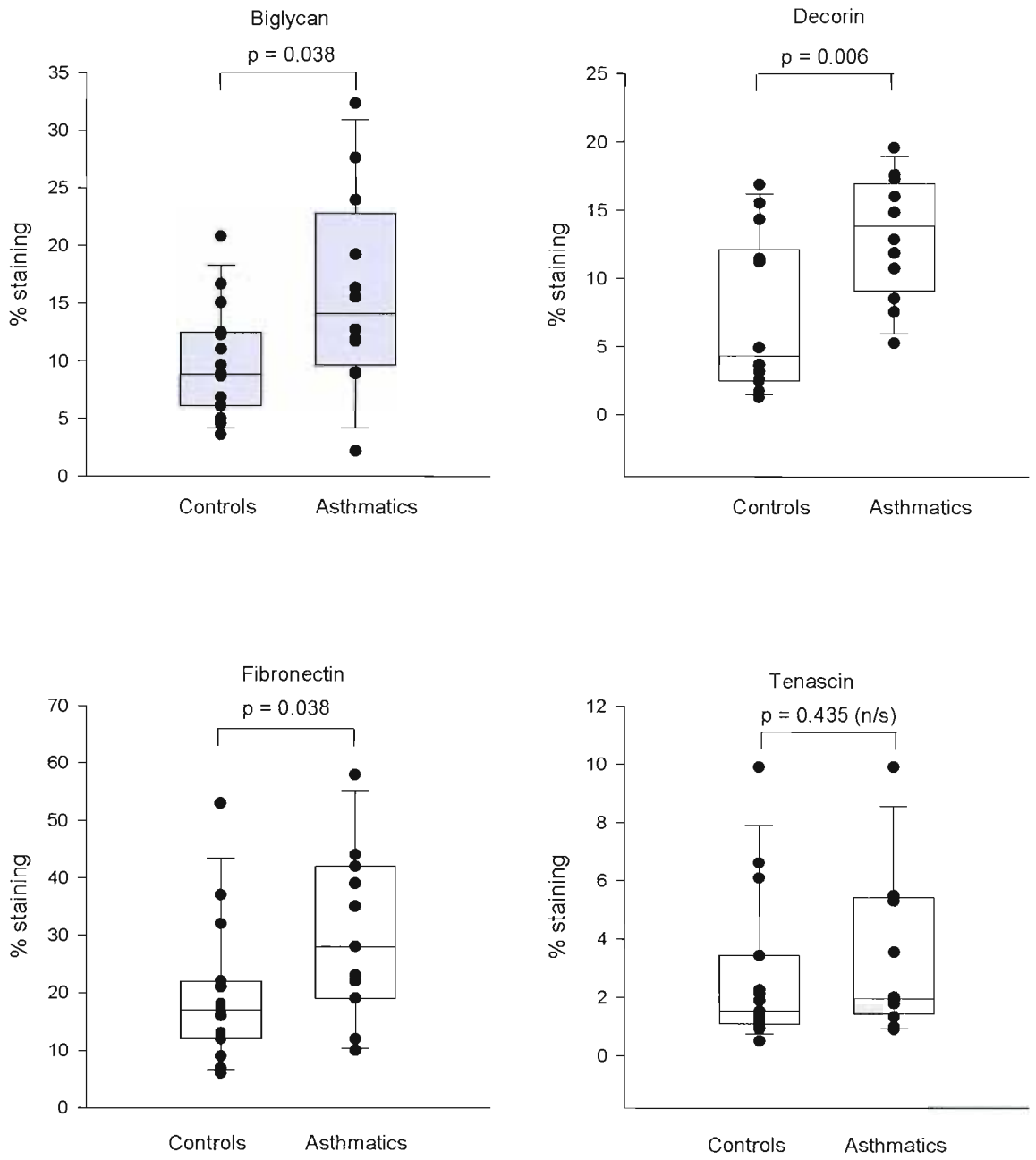


Figure 6-13 Proteoglycans in mild/moderate asthmatics and controls

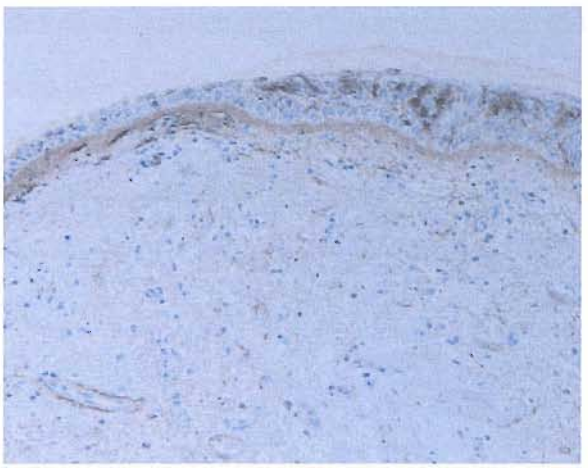


Figure 6-14 Example of an endobronchial biopsy stained for tenescin from an asthmatic subject

6.4.6 Airway wall thickness and eosinophils

An inverse relationship was seen between measures of AWT and eosinophils in the submucosa which just reached significance for the AWT parameter T/D. See Figure 6-15.

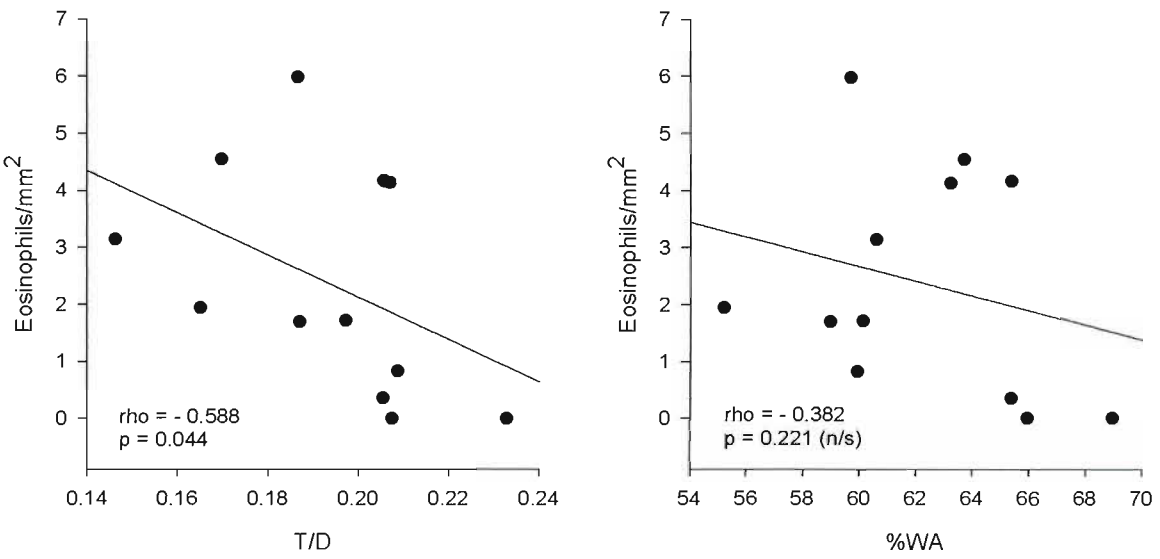


Figure 6-15 Regression analysis of submucosal eosinophils in mild/moderate asthmatics and AWT

6.4.7 Airway wall thickness and reticular basement membrane thickness

No significant relationship was found between measures of AWT and RbM thickness. See Figure 6-16.

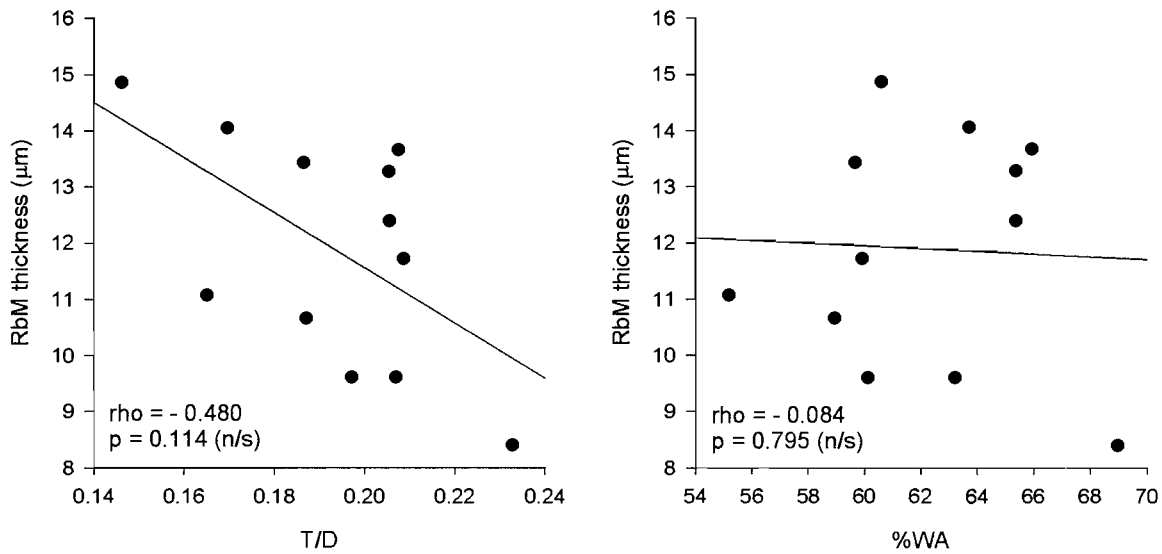


Figure 6-16 Regression analysis of RbM thickness in mild/moderate asthmatics and AWT

6.4.8 Airway wall thickness and sub mucosal collagens

A significant negative correlation was found between measures of AWT and collagen I staining of the submucosa in biopsies from mild/moderate asthmatics. No significant relationship was found between measures of AWT and staining for collagens III and V. See Figure 6-17.

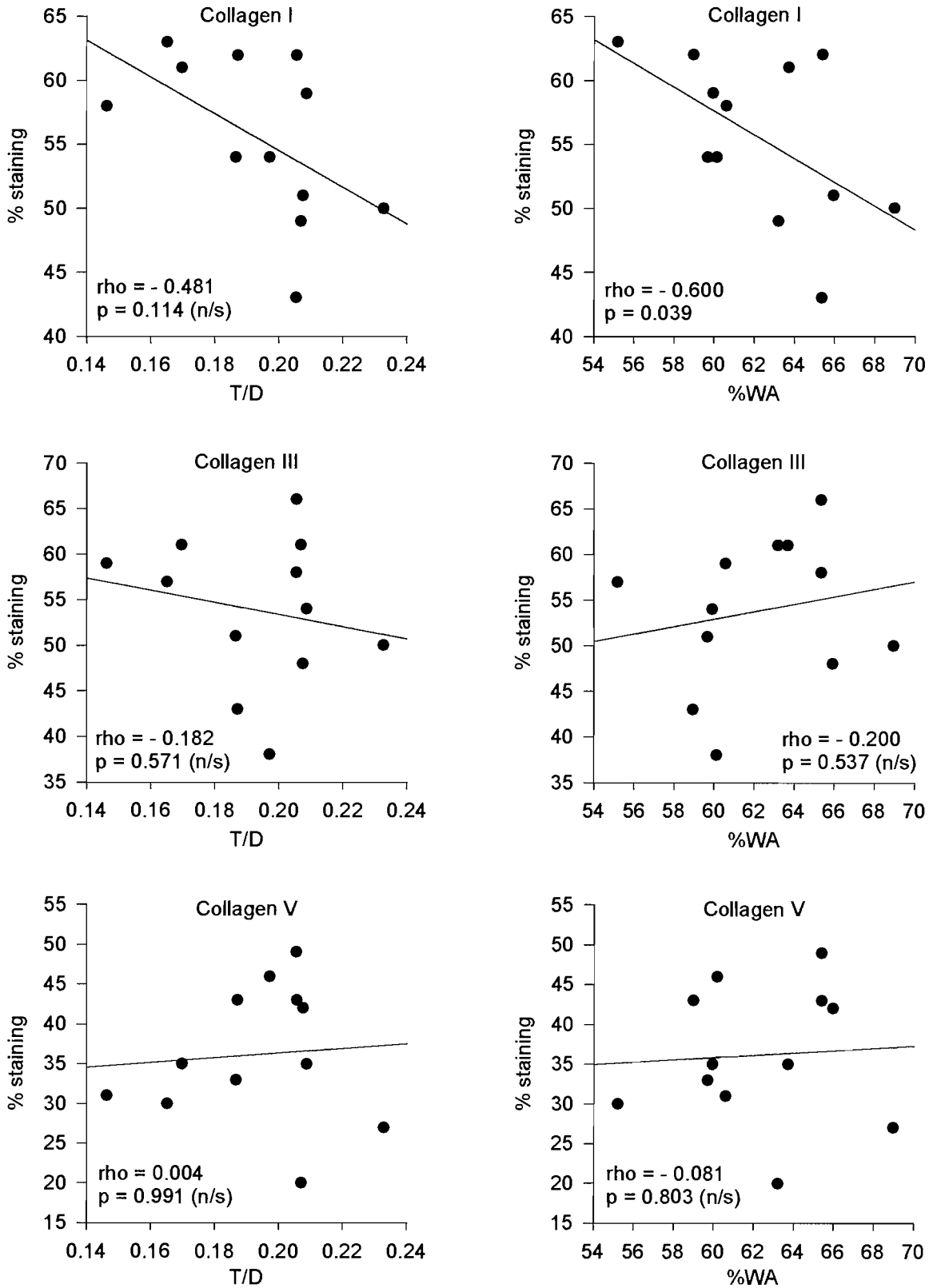


Figure 6-17 Regression analysis of submucosal interstitial collagens in mild/moderate asthmatics and AWT

6.4.9 Airway wall thickness and sub mucosal proteoglycans

A negative association was found between T/D and fibronectin but no significant relationship was found with the other proteoglycans, perlican, biglycan, decorin and tenascin in biopsies for mild/moderate asthmatics. See Figure 6-18.

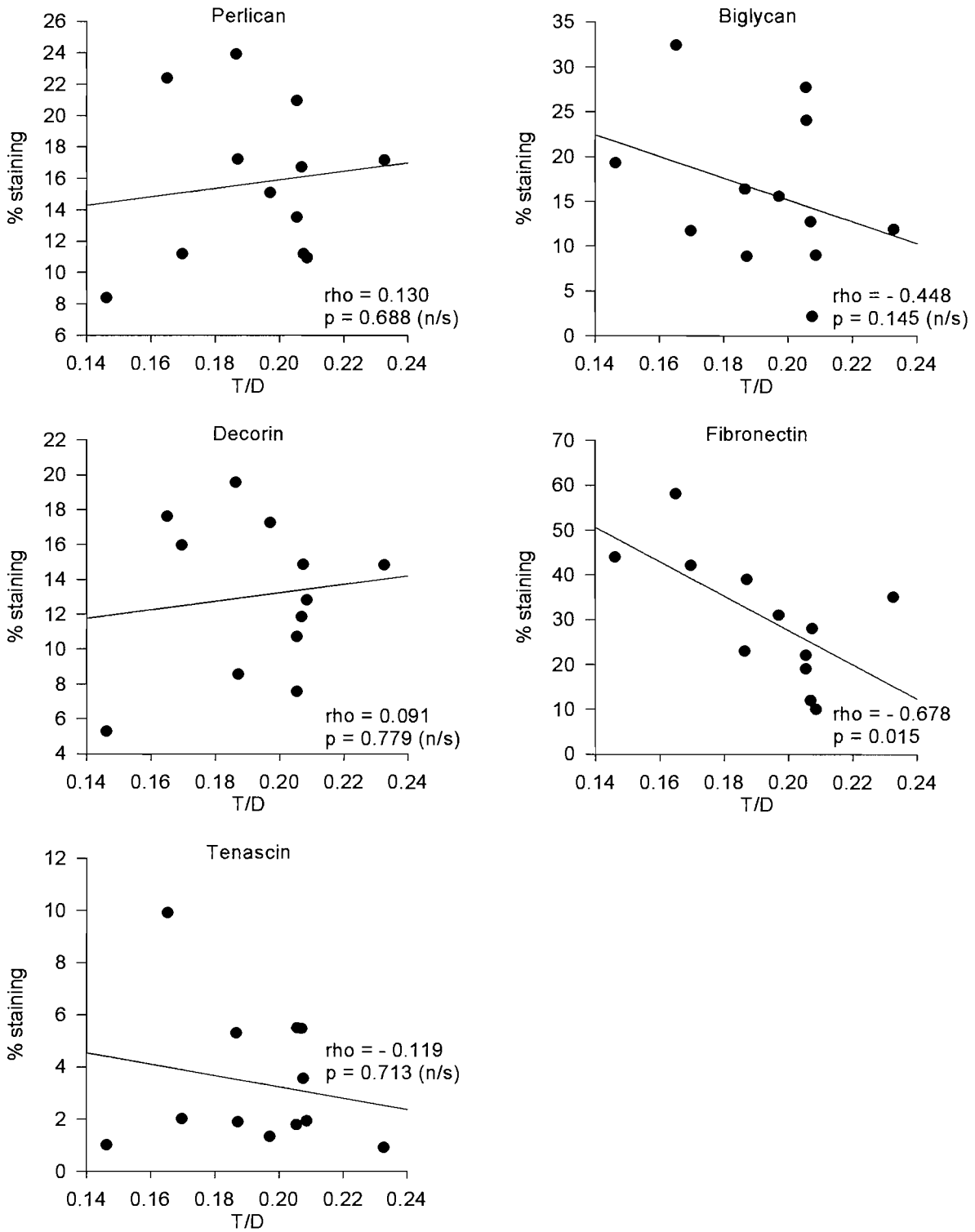


Figure 6-18 Regression analysis of submucosal proteoglycans in mild/moderate asthmatics and AWT

6.4.10 Bronchial hyperresponsiveness and reticular basement membrane thickness

No significant association was found between BHR in the mild/moderate asthmatics and submucosal RbM thickness. See Figure 6-19.

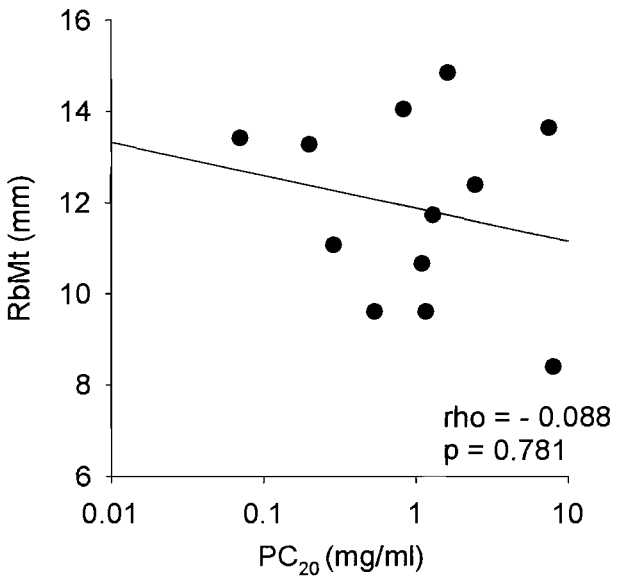


Figure 6-19 Regression analysis of PC_{20} (histamine) and RbM thickness in mild/moderate asthmatics.

6.4.11 Bronchial hyperresponsiveness and sub mucosal collagens

No significant associations were found between BHR in the mild/moderate asthmatics and submucosal staining for collagens I, III or V. See Figure 6-20.

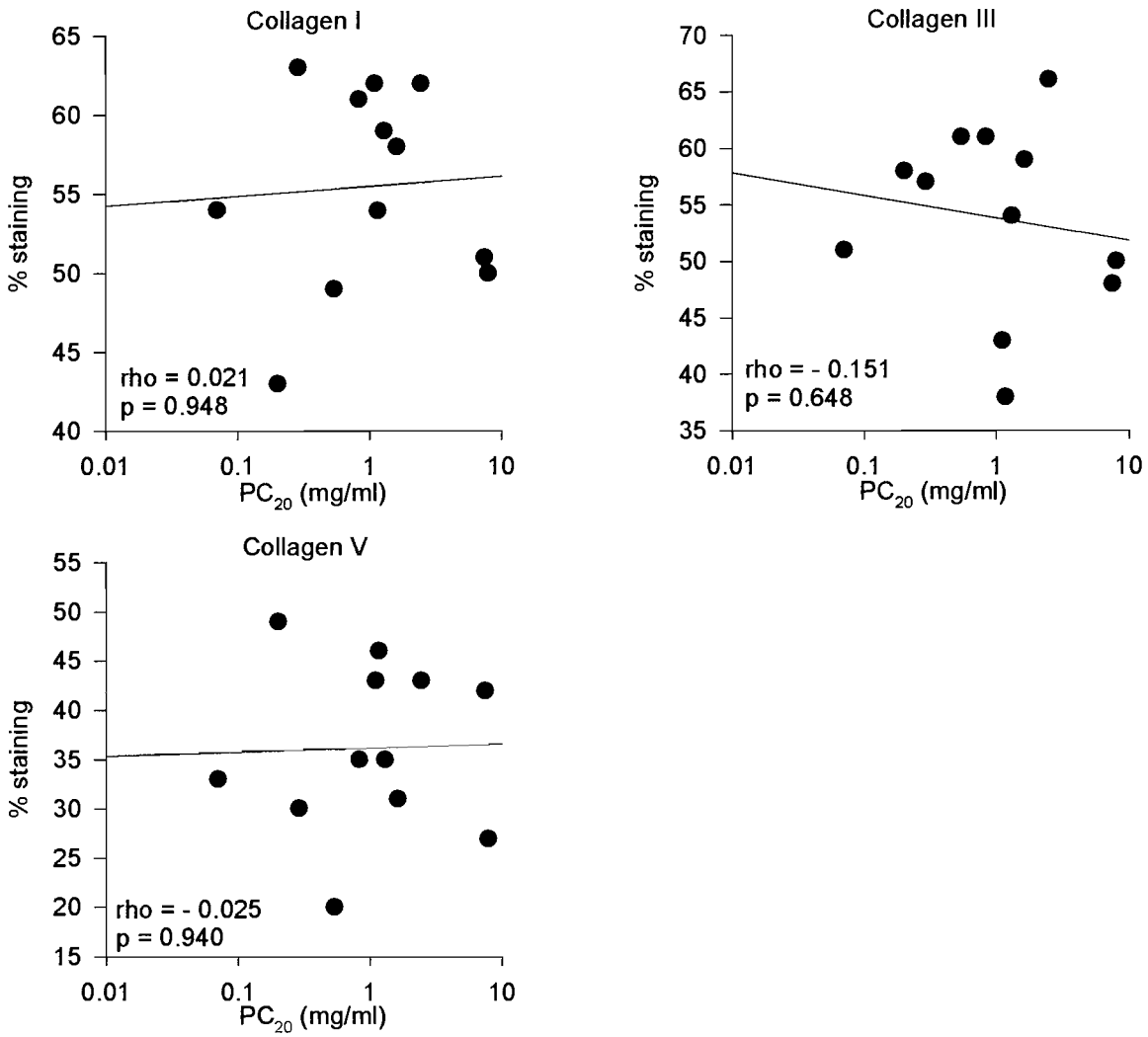


Figure 6-20 Regression analysis of PC_{20} (histamine) and submucosal collagens in mild/moderate asthmatics.

6.4.12 Bronchial hyperresponsiveness and sub mucosal proteoglycans

A significant negative correlation was found between submucosal perlican and BHR in the mild/moderate asthmatic group whilst biglycan and tenascin showed a similar but non significant trend in the same direction. See Figure 6-21.

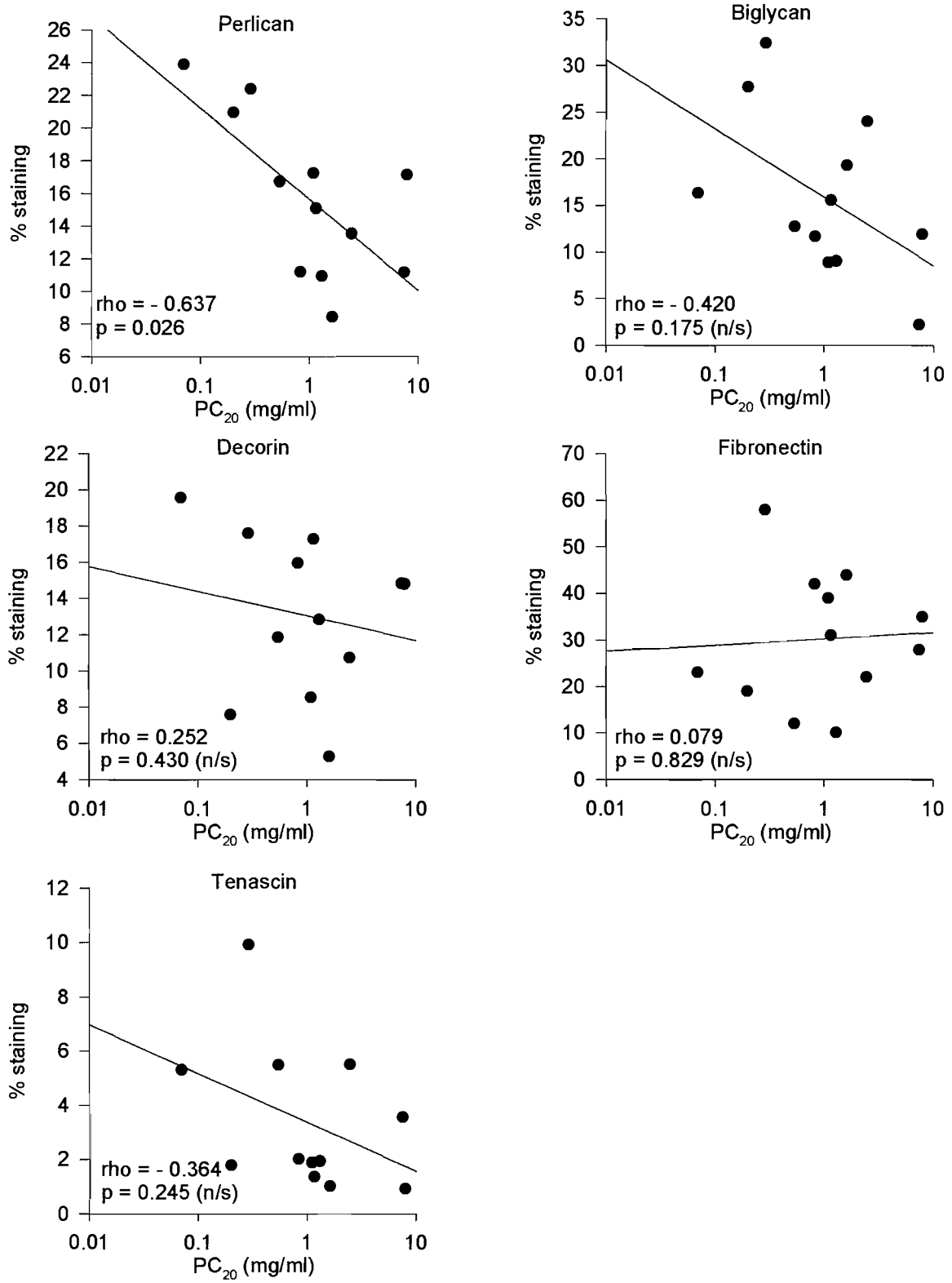


Figure 6-21 Regression analysis of PC_{20} (histamine) and submucosal proteoglycans in mild/moderate asthmatics

6.5 Discussion

6.5.1 Summary of results

Eosinophil numbers were greater in mucosal biopsies from asthmatic subjects compared to controls and this was most marked in steroid naïve asthmatic subjects. RbM thickness was increased in asthmatic subjects, with the usage of corticosteroids making no apparent difference to the results. Whole biopsy αSMA staining was not found to be different in asthmatic and control subjects. Collagens I, III and V were increased in the sub mucosa of asthmatic subjects, and in the case of collagens III and V this was positively associated with disease severity. The sub mucosal levels of the proteoglycans perlican, biglycan, decorin and fibronectin, were increased in asthmatic subjects but tenascin showed no difference between the two groups.

Amongst the mild/moderate asthmatic subjects a weak inverse relationship was seen between measures of AWT and eosinophil numbers in the submucosa which reached significance for the AWT parameter T/D. No significant relationship was found between measures of AWT and RbM thickness or submucosal staining for collagens III and V. Collagen I had a negative association with both measures of AWT, which reached significance for the AWT measurement %WA. Fibronectin staining of the submucosa in mild/moderate asthmatics was inversely related to airway T/D, but no relationship was seen with other proteoglycans. A significant negative correlation was found between submucosal perlican and BHR and biglycan and tenascin showed a similar but non significant trend.

6.5.2 Airway eosinophilic inflammation

The increase in eosinophils in asthmatics compared with controls was expected and has been well described previously in studies using similar bronchial biopsy techniques (52, 54, 55, 71, 210). These changes have been shown to extend into the peripheral airways on transbronchial biopsies (132, 211, 212) and can be reduced by treatment with oral or inhaled corticosteroid medication (58, 59, 65, 203, 213, 214). Although eosinophil levels are said to correlate with disease severity (52), the compounding factor of corticosteroid treatment probably explains the lack of an increase in eosinophils in the steroid treated mild/moderate asthmatic group, though it was observed in subjects using bronchodilators alone.

Inhaled corticosteroids have a greater effect on eosinophilic airway inflammation than they do on airway remodelling (195). Since the majority of asthmatic subjects in this study were taking these medications this may explain the inverse correlation seen between eosinophil numbers in the submucosa and AWT.

The significance inverse relationship between the AWT parameter T/D and submucosal eosinophils is interesting. This could be explained if a neutrophilic type of inflammation is present in the more severe asthmatics rather than eosinophilic as has been suggested by some authorities (215). Eosinophils are known to release proteolytic enzymes and this mechanism could therefore reduce AWT through degradation of the ECM (216). Alternatively those asthmatics with thickened airways may have been prescribed higher doses of inhaled corticosteroids with a corresponding suppression of eosinophils. The small subject numbers of asthmatics treated with and without inhaled corticosteroids make confident conclusions difficult.

6.5.3 Markers of remodelling in asthmatics and controls

Thickening of the RbM is the most commonly described marker of airway remodelling in asthmatics. An increase in the depth of this layer by 1.5 – 2.5x has been described in studies of bronchoscopic biopsies using similar techniques to those described in this study (70, 89, 96). The median thickness in this study was 8μm in the controls and 12μm in the asthmatic group, a difference of 1.5 fold. The subjects in this study all had relatively mild disease, which may explain why the difference is at the lower end of this range. Though whether RbM thickening in asthma is a marker of disease severity or just a marker of disease presence is disputed. Chetta *et al* described a significant relationship between RbM thickness and severity, based on a "current symptoms" score (97). I did not find such a relationship with severity and this view is supported by the work of Chu *et al* (101). Unfortunately in my study the numbers of subjects in each group were small and disease severity only categorised according to medication use rather than symptoms score, and therefore a significant relationship could have been missed.

An increase in smooth muscle in asthmatic airways in well described, both due to hyperplasia and hypertrophy (74, 217). The immunohistochemistry techniques used in this study with antibodies to αSMA demonstrate smooth muscle cells and submucosal myofibroblasts, and the latter are also increased in asthmatics (96). It is therefore perhaps surprising that increased αSMA staining was not observed in the biopsies of

asthmatics in this study. This may be attributed to the superficial nature of the bronchoscopic biopsies employed which do not provide a representative sample of deeper submucosal structures such as bronchial smooth muscle. It has however been reported that ASM in asthmatics extends more superficially than in controls (9). There was no significant different difference ($p = 0.224$) in the mean biopsy size from asthmatic ($0.72mm^2$) or control ($0.59mm^2$) subjects.

Immunohistochemistry analysis has demonstrated an increase in structural collagens in the submucosa as well as the RbM layer (70, 100, 101). The results in this study are in keeping with these previously published findings, including an increase in deposition with disease severity (100, 218, 219), though this has not been reported in all studies (101).

An increase in submucosal proteoglycan deposition is recognised in asthmatics and my results are in accordance with previously published work. Roche *et al* in their landmark study describing RbM thickening also demonstrated an increase in fibronectin in asthmatics (70). Huang *et al* described an increase in the small leucine rich proteoglycans lumican and biglycan, and the larger chondroitin sulphate related proteoglycan, versican in bronchoscopic biopsies taken from mild atopic asthmatics when compared with normal control subjects (114). Similar finding were described by Westergren-Thorsson *et al* for perlican, lumican, versican, hyaluronan and biglycan using tissue culture techniques and radio labelling of the different proteoglycans (113). I did not find a difference between submucosal tenascin staining in asthmatics in controls. An increase in tenascin has been described in asthmatics, but the deposition was largely confined to the RbM region, which was excluded in my analysis of the submucosa (98).

6.5.4 Airway wall thickening and markers of remodelling

One previously published study reported a significant correlation between RbM thickness and HRCT measurements of total airway thickness in a group of 49 mild/moderate asthmatics (140). In this study subjects were treated with oral corticosteroids for 2 weeks before measurement of AWT in order to minimise reversible airway inflammatory changes. I did not find such a relationship between total wall and RbM thickness and it is possible that this is because my assessments included remodelling and inflammatory changes both of which contribute to AWT. A recent paediatric study also found no relationship between AWT and RbM thickness (220).

It would be reasonable to hypothesise that increased airway thickness is due to a greater deposition of extracellular matrix materials. My results do not support this theory and indeed collagen I and fibronectin even showed a significant inverse correlation with AWT. The methods used determined density of staining in the submucosa of shallow biopsy samples and this may not reflect total collagen/proteoglycan deposition throughout the airway walls. A greater cellular infiltrate or oedema would also cause a reduction in staining, "by dilution", and proteoglycans are known to play a role in inflammatory control mechanisms (115) and be positively correlated with bronchial hyperresponsiveness (113, 221, 222).

6.5.5 Bronchial hyperresponsiveness and markers of remodelling

Several previous studies have described a positive correlation between bronchial hyperresponsiveness and RbM thickness in asthmatic subjects (71, 90, 195). Similar finding were shown in an animal model using ovalbumin sensitised Brown Norway rats (223). In this study the investigators also described an increase in collagen in the submucosa. I was unable to replicate these findings, possibly because of the small number of subjects or variations in inflammatory treatment between subjects.

A number of studies have demonstrated a positive correlation between submucosal proteoglycans in the airways of asthmatics and bronchial hyperresponsiveness. Huang *et al* studied bronchial biopsies from mild atopic asthmatics using immunohistochemistry techniques on frozen sections. Positive staining was corrected for basement membrane length, rather than submucosal area, and was found to correlate with BHR for lumican, biglycan and versican (114). Westergren-Thorsson *et al* cultured fibroblasts from steroid naïve mild asthmatics and quantified radio labelled proteoglycan production. They described a significant correlation between perlican, lumican, versican, hyaluronan and biglycan production and BHR (113). I found a significant correlation between BHR and perlican, but not with lumican, decorin, fibronectin or tenascin. A similar trend was seen between BHR and biglycan, but this did not reach statistical significance. My methods differ from those described by Westergren-Thorsson *et al* since they studied solely proteoglycan production, whereas my immunohistochemistry methods reflect the balance of production minus degradation. There are other factors that could influence these findings.

Positive staining is dependent on an antibody binding to a recognisable epitope. The primary immunohistochemistry antibody used for perlican was of the monoclonal type, whilst all other proteoglycans were stained with polyclonal antibodies, which may have improved the accuracy of the assay technique in assessment of perlican deposition in the submucosa. Analysis techniques using ELISA and molecular genetics, such as those described by Westergren-Thorsson *et al* above may be less susceptible to this variation.

6.5.6 The extra cellular matrix and control of airway thickening

Structural collagens and proteoglycans not only have a physical effect on airway wall mechanics but also play a role in modulation of inflammation by altering their binding of various cytokines and growth factors. Perlican is able to regulate RbM permeability and binds growth factors such as bFGF, PDGF, TGF-β, IL-4, IL-8, IFN-γ and GM-CSF (224). Similarly the profibrogenic factor TGF-β has been shown to bind to decorin, biglycan and fibronectin, demonstrating a mechanism by which the ECM can act as a reservoir for growth factors, allowing their release in response to appropriate stimuli (115, 225). TGF- β has been shown *in vitro* to stimulate fibroblasts to secrete ECM proteins, including collagens I and III, fibronectin, tenascin and proteoglycans (118, 221). Basal levels of TGF-β are increased in BAL from asthmatics, and may be further increased by allergen exposure (226). TGF-β mRNA expression also been shown to correlate with disease severity and RbM thickness (218).

Collagen homeostasis in the extracellular matrix is controlled by the balance of matrix metalloproteinases (MMPs), primarily MMP-9, and their inhibitors, the tissue inhibitors of metaloprotinases (TIMPs), primarily TIMP-1. MMP-9 present in the submucosa have the ability to degrade many ECM proteins, including collagens and proteoglycans, liberating bioactive fragments, that may alter cellular behaviour (227). In patients with asthma, MMP-9 is increased in BAL fluid in stable disease (228) and after local allergen challenge (229). TIMP-1 has fibrogenic properties and an increase in the TIMP-1 / MMP-9 ratio has been observed in asthma, being negatively correlated with FEV_1 (230) and positively associated with airway wall thickeness (measured using HRCT techniques) (231). An imbalance between MMP-9 and TIMP-1 activity in the submucosa has a major role in the pathogenesis of ECM remodelling and airflow limitation.

6.5.7 Limitation of methods used to study remodelling

Care must be taken in drawing conclusions from results where multiple comparisons have been made. Examples of this problem are seen in the coloration analysis of AWT with submucosal collagens and proteoglycans (figures 6-17 and 6-18). Since a p value of 0.05 is taken to indicate statistical significance, 1 in 20 times this is expected by chance alone.

Analysis of immunohistochemistry slides is semi-automated, involving the use of prewritten software macros, but remains subject to operator variability. Due to limitations of time, no assessment was made of reproducibility of the methods used in this study.

The numbers of subject studied was small and significant differences may have been missed as a result. Treatment regimens were varied, in particular with respect to corticosteroid use and this in likely to effect collagens and proteoglycan deposition.

Immunohistochemistry staining necessarily involves the study of thin sections that may not be representative of the airway as a whole. In particular there are many processing steps when compared with the direct integrated assessment of the whole airway wall using EBUS.

6.5.8 Conclusions

As expected eosinophilic inflammation and airway remodeling, as measured by RbM thickness and submucosal collagen and proteoglycan deposition, was shown to be present in the group of mild/moderate asthmatics when compared with control subjects. In the case of collagens III and V this was positively associated with disease severity. No consistent relationship could be shown between AWT and RbM thickness, sub mucosal collagens or proteoglycans. No significant relationship was found between BHR and RbM thickness, sub mucosal collagens or proteoglycans.

7 General Discussion

7.1 Summary of findings

The work in this thesis has lead to the following findings:-

- Endobronchial ultrasound using a radial probe is a valid tool for the measurement of wall thickness in central cartilaginous airways in an animal model. Inflation of the saline filled balloon sheath does not cause a significant degree of airway distortion.
- Measurements of AWT using endobronchial ultrasound demonstrate a significant increase in wall thickness and area in mild to moderate asthmatics compared with healthy non-asthmatic controls. The use of EBUS in this way has not been described previously in the published literature.
- Assessments of wall thickness of the same airway by EBUS and HRCT give similar values and my results are in broad agreement with previously published data. Inter and intra observer variability in EBUS image analysis is within acceptable limits. AWT of similar sized airways shows little variability between different sites within the lung suggesting that measurement in one area of the lung can be taken as representative of the lung as a whole.
- Bronchial hyperresponsiveness is inversely correlated with AWT in asthma. Airway thickening alone therefore cannot be an important determinant of bronchial hyperresponsiveness as suggested by mathematical modelling.
- AWT is negatively correlated with reversibility to β_2 agonists.
- Reticular basement membrane thickness, submucosal collagens I, III, V and proteoglycans perlican, biglycan, decorin, fibronectin, but not tenascin, are increased in asthmatic subjects. In the case of collagens III and V there evidence of a relationship with disease severity. No difference was observed in smooth muscle (αSMA) area between bronchial biopsies from asthmatic and control subjects.
- No significant relationship was shown between measures of AWT and RbM thickness or sub mucosal levels of collagens III and V. Collagen I had a negative association with both measures of AWT, which reached significance for the AWT measurement %WA. Fibronectin staining of the submucosa in mild/moderate asthmatics was inversely related to airway T/D, but no relationship was seen with other proteoglycans. A negative association was found between submucosal

perlican and BHR and biglycan and tenascin showed a similar but non significant trend.

- Eosinophil numbers are increased in the submucosa of asthmatics and this is most marked in steroid naïve subjects. In mild/moderate asthmatics eosinophil numbers in the submucosa are positively correlated with the AWT parameter T/D, but %WA did not reach statistical significance.

7.2 Other findings

It is tempting to identify the changes associated with remodelling as airway "scarring", resulting from a prolonged period of uncontrolled inflammation, and the cause of the accelerated decline in FEV_1 and diminution of full reversibility to bronchodilators and anti-inflammatory agents seen in some asthmatics (12, 232). Although a convenient explanation, these links are far from proven and indeed in this study no significant association could be found between AWT and asthma duration, post bronchodilator FEV_1 or RbM thickness. Whilst some previously published studies have reported an inverse relationship between AWT and FEV_1 (139, 140, 179), others have found no relationship consistent with the results described here (138, 142). Only one published study has examined the relationship between whole AWT (measured using HRCT) and RbM thickening and found these measures paralleled each other (140). As single published study has demonstrated an inverse relationship between AWT and duration of disease (233). The failure in this thesis to demonstrate significance correlations between AWT and the parameters mentioned above may also result from a lack of statistical power due to the relatively small number of subjects studied.

7.3 Functional consequences of airway remodelling

Airway calibre *in vivo* is the results of a delicate balance between the force generated by ASM contraction and a number of opposing forces. Since airways do not exist in isolation but are embedded in a complex meshwork of parenchymal supports these opposing forces are not just affected by local wall tensions, but also coupled loads from other airways and alveoli as a result of interdependence across the whole lung (234).

There are three possible explanations that can account for the greater propensity for airway narrowing in asthmatics that is bronchial hyperresponsiveness. These proposals are not mutually exclusive:-

- Firstly airway smooth muscle in asthmatics could contract with a greater force than in controls, either because of an innate property of the muscle fibres themselves or as a result of their greater numbers.
- Secondly the force of muscle contraction could remain the same, but a reduction in muscle load or opposing forces would lead to an increase in fractional shortening and hence airway narrowing.
- Thirdly, as proposed by mathematical modeling, an increase in the lamina propria, internal to the smooth muscle layer due to oedema, cellular infiltrate or both, could augment airway narrowing for the same degree of smooth muscle shortening.

The interactions of factors that favour and oppose airway narrowing are complex and are summarised in Figure 7-1.

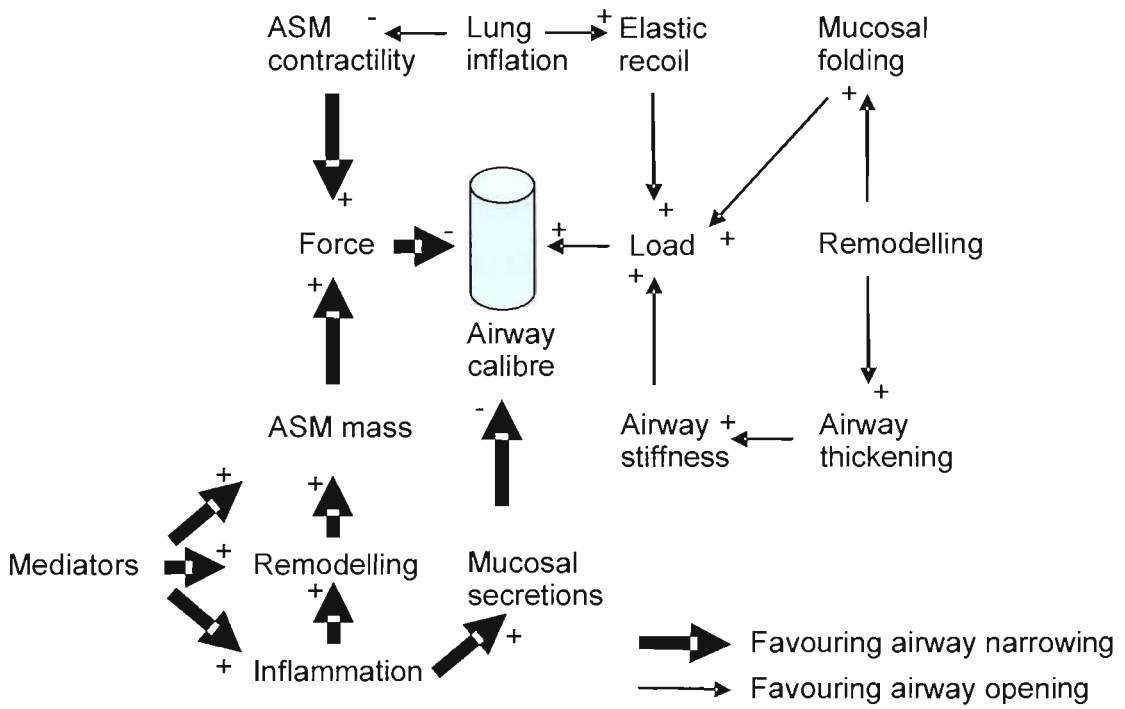


Figure 7-1 Schematic diagram representing the complex interrelationship between factors that favour and oppose airway narrowing
(modified from ref (235))

7.3.1 Force of airway smooth muscle contraction

Even from the earliest days of asthma research smooth muscle has been identified as the key effector of airway narrowing. It seems intuitive to assume that in asthmatics the narrowing is as a result of a greater force of airway smooth muscle contraction. In reality *in vitro* experiments have shown that explanted bronchial or tracheal smooth muscle tissue can demonstrate increased (236, 237), normal (237-239) or even reduced isometric force generation (240, 241). More recently it has been shown that isometric force and velocity of shortening can be increased by passively sensitising ASM with serum from asthmatics containing high levels of IgE (104, 242) and that ASM from sensitized asthmatics may be phenotypically different, containing a different balance of myosin light-chain kinase isoforms (243, 244).

The finding that ASM from asthmatics may exhibit a greater maximum velocity of shortening gave rise to a theory that this could explain BHR (105). If ASM is routinely stretched during the normal pattern of intermittent deep inspirations, but returns more rapidly to its contracted state during expiration in asthmatics then this could result in greater narrowing and BHR. In support of this theory, Skloot *et al* observed that BHR in normal subjects prevented from taking a deep breath during a methacholine challenge test was similar to that seen in asthmatics, but that this apparent responsiveness fell substantially when they, but not in the asthmatics, inhaled to TLC during airflow obstruction assessment (245).

Post mortem studies demonstrate an increase in ASM mass in the airways (74, 217), and as a result it could reasonably be expected to generate more total force for airway narrowing (89, 128). However not all *in vitro* preparations have confirmed that an increase in mass is associated with an increased force generation capacity, in ASM from pulmonary arteries (246) or asthmatic airways (247). It has been suggested that the increase in ASM in asthmatic airways shown in post mortem studies could have been overestimated. Thomson *et al* examined large central cartilaginous airways using stereology, which eliminates the shrinkage effects of tissue fixation, and found no increase in ASM in asthmatics (248). It must be remembered that changes in ASM mass do not occur in isolation and alterations in the composition of the surrounding extracellular matrix may counteract these forces. Indeed there is evidence to suggest that ASM may influence its own environment/proliferation through mediator and ECM proteoglycan release (249).

7.3.2 Airway smooth muscle loading

The resultant airway narrowing depends on the net effect of ASM force minus the load against which this force acts (234). The opposing forces on ASM can act either in compressive (on structures in inner layer of the airway), in parallel (due to ECM material between ASM bundles) or in a serial manner (due to tension from parenchymal support attachments). The mechanical properties of ASM are such that, if unimpeded, it may shorten by up to 70% in *in vitro* preparations (186). *In vivo*, such a shortening would result in complete airway closure (124). Which suggests that mechanisms exist to oppose ASM shortening (103).

As an indirect non invasive index of airway stiffness, studies have examined the change in anatomical dead space with lung volume during inspiration. Using this method the change in dead space has been found to be reduced in asthmatics (232, 250). Asthmatic airways have also been shown to be less collapsible during forced expiration (251, 252). This evidence is suggestive of increased stiffness in asthmatics but does not confirm this change is as a result of remodelling.

Evidence is available from animal models to link airway stiffening, remodelling and in the longer term a reduction in BHR. In a study using ovalbumin (OA) sensitised rats Palmans *et al* demonstrated that after 2 weeks of OA exposure, airway histology revealed goblet-cell hyperplasia, an increase in fibronectin deposition, and a thickening of the airway inner wall area associated with an increase in airway hyperresponsiveness to aerosolized carbachol. After further OA exposure to 12 weeks the increases in submucosal fibronectin and collagen remained but airway hyperresponsiveness progressively declined to the level seen in the control group by the end of the study. This supports the view that prolonged allergen exposure may can enhance extracellular matrix deposition and protect against bronchial hyperresponsiveness (223).

Bundles of collagen fibres run parallel to ASM cells, in a circumferential pattern around the airway and are increased in number in asthmatics (100). These fibres in which the ASM is embedded are non-compressible, therefore during contraction they fold and provide a parallel afterload resisting further airway narrowing. A similar increase is seen in submucosal proteoglycans (113, 114). The results described in chapter 6 are also in agreement with these previously published findings. Figure 7-2 demonstrates the increased submucosal collagen I staining in asthmatics when compared to controls and Figure 7-3 shows how the collagen is intimately wrapped within and around ASM

bundles in asthmatics. This demonstrated the interdependence of these structures in enhancing and opposing airway narrowing.

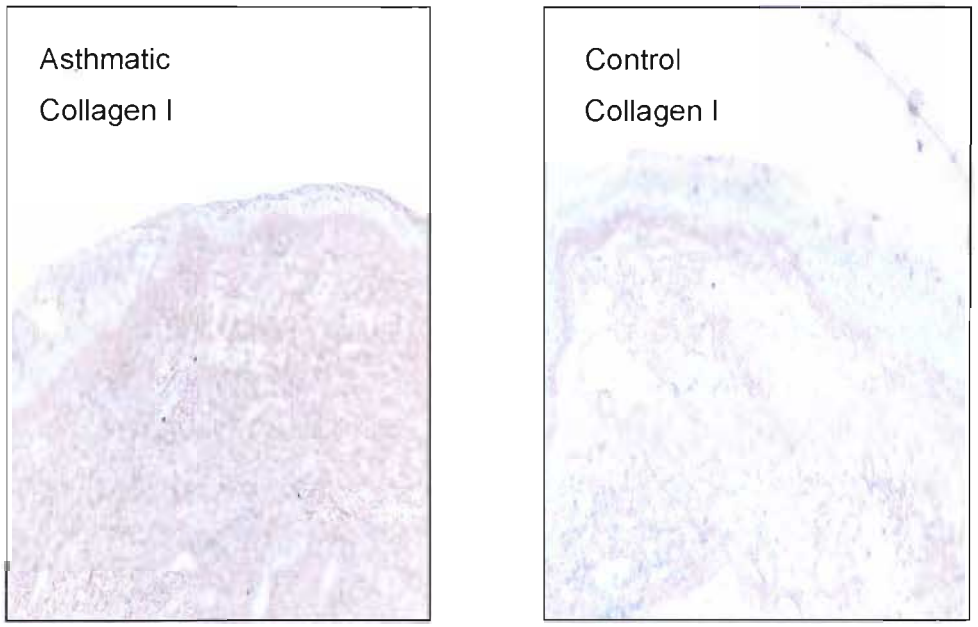


Figure 7-2 Examples of collagen I staining in asthmatic and control biopsies

Figure 7-3 Comparison between αSMA and collagen I staining in biopsies from asthmatics

In contrast to the increase in collagens and proteoglycans, elastic fibre numbers have been shown to be decreased in asthmatics, though it is not fully understood whether this is due to mechanical fragmentation or enzyme degradation (111). A reduction in tissue elastance leading to reduced ASM loading has also been proposed as an

explanation for enhanced airway narrowing in asthma. Bramley *et al* studied full thickness, circumferentially cut, lobar bronchial preparations obtained from one asthmatic and six non asthmatic lobectomy subjects. No difference was observed in cross sectional smooth muscle area but isotonic shortening was dramatically greater in the preparation from the asthmatic subject (31 vs 11%) (247).

When ASM cells contract their volume is conserved and they become "shorter and fatter". If the muscles ability to "fatten" is constrained by a thickening of its surrounding connective tissue matrix then this will act to oppose contraction (248). In an attempt to demonstrate this Meiss *et al* placed stiff silastic bands around explanted canine tracheal smooth muscle and found this reduced maximal shortening by ~15% (253).

The concept that parallel elastic impedance and/or radial constraint, by structures surrounding ASM bundles, can attenuate shortening has been supported by the observation that ASM shortening increases after treatment with collagenase. Bramley *et al* studied fractional shortening before and after incubation with collagenase in human ASM preparations and found this treatment increased the degree of shortening by 5 and 9% with electrical and histamine stimulation respectively (254). In an extension to the study by Meiss *et al* described above collagenase treatment again increased ASM shortening in canine tracheal ASM preparations (253). An explanation for the effects described is that collagenase digestion removes the radial constraint and parallel elastic impedance, thereby allowing the unloaded ASM to achieve a greater degree of shortening.

A serial elastic load is also applied against ASM contraction by parenchymal supports attached to the external adventitia around the airway. Bronchi distend as the lungs are inflated as a consequence of the decrease in intra pleural pressure transmitted via interdependent adventitial supports. In a complex theoretical analysis Macklem *et al* concluded that the degree of ASM loading decreases with lung volume and transmural pressure. Any peribronchial inflammation would enable partial uncoupling of the ASM from this parenchymal recoil, which may be an important mechanism producing excessive bronchoconstriction in asthma (234). This principle of uncoupling has been artificially induced in dogs by rapid saline infusion. This was shown to accentuate histamine induced bronchoconstriction, without causing a change in outer airway area on HRCT. Quantitative modelling indicated that the oedema in the airway wall was mostly outside the smooth muscle (193).

7.3.3 Thickness of the inner airway wall

The thickness of the inner airway wall, which consists of the epithelium, RbM, lamina propria and submucosa, lies internal to the ASM layer (255) and present an additional serial load to bronchoconstriction. The inner layer is incompressible and during bronchoconstriction the mucosal margin is thrown into folds that undergo regions of tensile and compressive stress (256). RbM thickness has been shown to be inversely correlated with BHR (as a clinical marker of airway stiffness) in a study of asthmatics and patients with COPD (257). The bending stiffness of a layer is directly related to its thickness cubed, hence modelling predicts that thickening of the RbM will increase ASM serial loading (129). The same model also predicted that thickening of the inner layer should lead to a reduction in the number of mucosal folds, a change that has not been verified in asthmatic subjects *in vivo* (112).

Using another mathematical model Seow *et al* also proposed that the maximum shortening of ASM is inversely proportional to the number of folds and the thickness of the lamina propria – submucosal layer (258).

A summary of the mechanisms by which remodelling may exaggerate or inhibit maximal airway narrowing is shown in Table 7-1.

Structural change	Mechanism	Effect
Increased intraluminal secretions	Direct airway narrowing	Reduced baseline airway calibre
Thickening of inner wall area	Amplification of ASM shortening	Enhanced narrowing for same ASM shortening
ASM hyperplasia/hypertrophy	Possible increase in contractile force	Enhanced ASM shortening and airway narrowing
Adventitial thickening in outer wall layer	Reduced ASM muscle load	Enhanced ASM shortening and airway narrowing
Reduced elastin in airway wall	Reduced ASM muscle load	Enhanced ASM shortening and airway narrowing
Cartilage degradation	Reduced ASM muscle loading in cartilaginous airways	Enhanced ASM shortening and airway narrowing
Thickening of RbM	Increased stiffness and ASM load to generate folding	Reduced ASM shortening and airway narrowing
Inner wall area increased collagen and proteoglycan deposition	Increased stiffness of subepithelial matrix and ASM load	Reduced ASM shortening and airway narrowing
Increased collagen and proteoglycan deposition around ASM bundles	Increased parallel loading to ASM and "banding" effects	Reduced ASM shortening and airway narrowing

Table 7-1 Mechanisms by which remodelling may affect maximal airway narrowing

7.4 Can airway remodelling be reversed?

There is growing evidence that airway remodelling can, at least partly, be reversed, though the degree and time course is probably different to changes in inflammation and BHR. Using an OA sensitised rat model Vanacker *et al* examined the effect of inhaled salmeterol, fluticasone or both on airway inflammation, remodelling and hyperresponsiveness. After 4 weeks OA exposure an increase in inflammatory cell numbers, goblet cells, fibronectin and collagen was observed. Inhaled salmeterol reduced inflammatory cell numbers but did not affect the structural changes, whilst inhaling both salmeterol and fluticasone reduced goblet cell numbers but increased deposition of collagen and fibronectin further. Despite the changes in the extracellular matrix no increase was observed in airway hyperresponsiveness. The authors suggested that remodelling may act to stiffen the airways and thus protect against allergen induced hyperresponsiveness (208, 209, 259).

There is evidence in human studies that airway remodelling is at least partly reversible when occupational exposure ceases. In a group of individuals who developed asthma following industrial exposure to toluene di-isocyanates it was observed that subepithelial basement thickening decreased 6-20 months after exposure stopped. This change was accompanied by a reduction in fibroblasts, mast cells and lymphocytes (260, 261).

A number of studies have demonstrated that intervention with inhaled corticosteroids is able to reduce RbM thickness, though higher doses and/or a longer duration of treatment appears necessary than is required to suppress symptoms or BHR. Hoshino *et al* carried out a randomised double blind study into the effect of inhalation of beclomethasone dipropionate (BDP) 400mcg bd. After 6 months of treatment they found a significant reduction in RbM thickness and submucosal vascularity as markers of remodelling (204, 206). In this same group of steroid treated patients the reduction in RbM thickness was paralleled by a fall in the number of submucosal fibroblasts, insulin like growth factor (IGF) -1 and expression of the matrix metalloproteinase MMP-9. Expression of the metalloproteinase inhibitor TIMP-1 was suppressed. The authors suggest that these biochemical changes may provide an explanation for the steroids mechanism of action (204, 205). Most other groups (98, 194, 201, 214, 262, 263), but not all (67) have also shown a small effect of ICS on reducing RbM thickness and this has been discussed in a number of review articles (15, 264, 265)

Ward *et al* utilised a high dose of the inhaled steroid fluticasone proprionate (FP) (750mcg bd) on a group of mild/moderate asthmatics. They found that whilst inflammatory cells, FEV_1 and BHR improved after 3 months but a reduction in RbM thickness was not evident until 12 month of treatment (195). Chetta et al examined the effect of a low (FP 100mcg bd) and high (FP 500mcg bd) doses of inhaled steroids on the vascular component of airway remodelling over a 6 week period. Whilst both dose regimens improved inflammatory cell numbers and bronchial hyperresponsiveness, only at the higher dose was an effect seen on the number of vessels, vascular area and RbM thickness (266).

Not all studies have demonstrated an effect of steroids on remodelling. Chakir *et al* treated mild, moderate and severe asthmatics with inhaled and oral corticosteroids and found no reduction in submucosal collagens I and III. The authors suggested this was due to raised levels of TGF-β which was similarly unaffected by treatment (219).

Two studies in the published literature have examined the effect of corticosteroid treatment on whole airway thickness by HRCT. Paganin *et al* studied 10 subjects before and after a 2 week course of parenteral high dose corticosteroids. In 4 subjects airway wall thickening was present before and after treatment, suggesting that the steroid treatment had no effect (267). Given the results of more recent studies the treatment period was probably of too short a duration and the study is further limited by the non quantitative analysis of the data. A more recent and quantitative study by Niimi *et al* examined the effect of 12 weeks treatment with BDP (800mcg/day) on 45 previously steroid naïve asthmatics. Following the treatment period AWT of the right upper lobe bronchus was modestly but significantly reduced compared to the baseline scan, though it remained greater than that seen in a group of control subjects (233). Although this is an important study it does not answer the question as to whether steroid treatment affects AWT by a reduction in inflammatory processes such as oedema, cellular infiltrate or mucous secretion or by an effect on airway remodelling. A further HRCT scan 4 weeks into treatment, after suppression of a supposed inflammatory but not a remodelling component of airway thickening could have helped answer this question. The repeated radiation exposure required for intervention studies of this type in asthma is difficult to justify ethically and the authors rightly only performed a very limited HRCT scan on just 2 occasions.

7.5 Airway remodelling is beneficial

It has generally been assumed that remodelling has a negative effect on patients with asthma. It has been held up as the cause of irreversible airway narrowing and ongoing bronchial hyperresponsiveness. There are however a number of possible benefits that remodelling could bring to protect the airways in asthmatics (268).

As previously described remodelling causes stiffening of the airways and this presents a load to protect against excessive airway narrowing in response to constricting stimuli and therefore BHR. It is interesting that clinically severe asthmatics typically fall into one of two types, with some patients exhibiting fixed airflow limitation, whilst others show persistent symptoms and significant diurnal variation in peak flow measurements despite treatment. It is tempting to hypothesise that the former group have more "remodelled" airways that are able to resist bronchoconstriction, whilst the later do not and are at particular risk of sudden death (269). The reasons behind the different clinical phenotypes are at present unclear.

Theoretically the increased vascularity of the airway wall that accompanies the remodelling process could facilitate removal of contractile agonists and inflammatory cytokines, although these same vessels are also the source of oedema fluid and inflammatory cells. Similarly, an increase in goblet cells and intra luminal mucous could afford protection against inhaled toxin and prevent mucosal dehydration.

7.6 Can EBUS quantify airway wall constituents?

EBUS has been shown to be a valuable tool in the measurement of whole AWT without radiation exposure or a requirement for resected tissue. It would be of even greater interest if it were possible to identify specific airway regions or structures, such as the lamina propria or ASM thickness. Is this ever likely to be possible? A Swiss research group recently approached this in a novel way by studying airways in a group of 10 lung transplant recipients. Sequential EBUS images, using a 20MHz radial probe, were obtained both proximal (i.e. from the recipient lung) and distal (i.e. from the donor lung) to the transplant anastomosis. This provided an elegant mechanism for "control" images to be obtained. The thickness of the first hypoechoic layer, which the authors ascribe to the submucosa, was increased in subjects with graft rejection. The authors concluded therefore that EBUS may be used to investigate and quantify inflammatory alterations of bronchial wall structures *in vivo* (178). I do not believe this study

demonstrates it is possible to reproducibly measure elements of the airway wall using current EBUS technology and that these correlate with inflammatory processes. No biopsies or other histological confirmation was obtained and their conclusion that the first hypo echoic layer equates to the submucosa (between the mucosa and inner cartilage boundary) is unjustified. A Japanese group reported a single case where subepithelial thickness was decreased in an asthmatic subject following treatment with montelukast (270). Again I am sceptical that the region being measured equates to a specific region of the airway wall. Where the EBUS balloon sheath is in contact with the airway wall a dense hyper echoic band is seen and this is known be significantly greater than the mucosal thickness (153, 160, 176, 177). It is an attractive idea to measure airway subcomponents and correlate them with markers of inflammation and remodelling, but I do not believe this is possible with the current generation of EBUS technology.

7.7 Future directions for study

This study is the first of its kind to use the novel technique of endobronchial ultrasound to measure AWT. As such it serves as a pilot study to prove the concept and provides a spring board from which to use the technology to study the airways further in asthma and other respiratory diseases.

7.7.1 Control of the inflammatory component of wall thickening

In this study subjects continued their usual asthma treatment, which did not always include inhaled corticosteroids. In order to reduce as far as possible inflammatory oedema and cellular infiltrate and just measure airway thickening due to remodelling, subjects should ideally be treated with a high dose of inhaled corticosteroids before measurement of BHR and assessment of airway thickness. This may make recruitment much more difficult, as treatment may reduce BHR to within the normal range, but will give more informed results as to the effect of thickening due to remodelling alone.

7.7.2 Airway wall thickness in severe asthmatics

The research described in this thesis included only mild/moderate asthmatics with most having a near normal post bronchodilator FEV_1. Further work is needed to extend the asthmatic subject group to include more severe patients, including those with fixed

airway obstruction to test the hypothesis that AWT is related to post bronchodilator FEV_1.

7.7.3 Automated airway assessment of airway wall thickness

The EBUS image analysis used in this study is operator dependent. Automated algorithm based image interpretation, as developed by Nakano (147) and Niimi (141) would improve consistency of results when measuring AWT.

7.7.4 Intervention studies

EBUS offers the possibility of AWT measurement without radiation exposure. This is a vital concern if a study is conducted into investigate the effect of an intervention by performing a baseline assessment followed by repeated measurements during a treatment period. A high dose of an inhaled steroids for 3 months, or the anti-IgE omalizumab, would be possible at the present time. EBUS could also be used in the assessment of new treatments for asthma in the future. Possible candidates would be anti TNFα drugs and if they can be shown to be safe, drugs that are developed to alter the process of remodelling, for example by altering the MMP 9/TIMP-1 axis.

7.7.5 Longitudinal studies

In the same vein, EBUS allows the possibility of serial airway measurements in subjects with asthma, atopic rhinitis and those with asymptomatic BHR. Do these subjects have a sub clinical level of airway thickening? Is airway thickening present in children with asthma and what happens to the airways in those who apparently grow out of the disease?

7.7.6 Detailed assessment of airway wall layers

Using the 20 MHz radial probe that is currently commercially available it is not possible to distinguish between the different layers within the airway. Resolution could be improved by increasing the frequency to 30-40 MHz since only limited penetration is required. Cartilage has a very strong echogenicity and this is likely to interfere with high resolution detail. If it is possible to differentiate the ASM layer then *in vivo* assessment of this and the thickness of the submucosa thickening become realistic for the first time. If the technology described can be validated in a clinical setting then the intervention studies described above would yield further important data.

7.7.7 Small airway wall thickness

In the study described in this thesis I examined a medium sized segmental airway of approximately 4-5mm in diameter. This required the use of a fluid filled balloon sheath to provide sonic coupling. If the probe is advanced into an airway of 2mm in diameter it would make direct contact with the airway wall and the fluid filled sheath would be unnecessary. This would also enable the assessment of smaller more peripheral airways where previous work is very limited. Although transbronchial biopsies have been performed in a research setting there are clinical risks with this procedure which are not present with EBUS. Although HRCT resolution is improving at present there is no prospect of this imaging modality permitting details wall thickness measurements in small airways of this size.

7.8 Final conclusions

EBUS has the potential to be a valuable tool in the assessment of AWT. It has been shown to be accurate and reproducible in different areas within the lung and repeatable between operators. In this thesis I have described how EBUS demonstrates total airway thickening in asthmatics. This increase in airway thickness is inversely related to BHR, in contradiction to that predicted by mathematical models bases on geometric principles. AWT has been shown to be accompanied by increases in structural collagens and proteoglycans in the submucosa. These materials are found in close association with the contractile elements within the airway walls and act as serial and parallel loads opposing airway narrowing and thereby reducing hyperresponsiveness. I suggest that airway remodelling may have a beneficial role by "stiffening" the airways, preventing excessive bronchoconstriction, and therefore protecting the airways from the clinical consequences of closure.

Chapter 8: References

8 References

1. Ciba Guest Foundation. 1959. Terminology, definitions and classification of chronic pulmonary emphysema and related conditions. *Thorax* 14:286-299.

2. American Thoracic Society Committee on Diagnositic Standards. 1962. Definitions and classification of chronic bronchitis, asthma, and pulmonary emphysema. *Am Rev Respir Dis* 85:762.

3. National Heart Blood and Lung Institute/World Health Organisation. 2002. Global INitiative for Asthma (GINA): Global Strategy for Asthma Management and Prevention NIH Publication No 02-3659. Bethesda.

4. Elias, J. A. 2000. Airway remodeling in asthma. Unanswered questions. *Am J Respir Crit Care Med* 161(3 Pt 2):S168-71.

5. Bousquet, J., P. K. Jeffery, W. W. Busse, M. Johnson, and A. M. Vignola. 2000. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 161(5):1720-45.

6. Fahy, J. V. 2001. Remodeling of the airway epithelium in asthma. *Am J Respir Crit Care Med* 164(10 Pt 2):S46-51.

7. Haahtela, T. 1997. Airway remodelling takes place in asthma--what are the clinical implications? *Clin Exp Allergy* 27(4):351-3.

8. Tiddens, H., M. Silverman, and A. Bush. 2000. The role of inflammation in airway disease: remodeling. *Am J Respir Crit Care Med* 162(2 Pt 2):S7-S10.

9. Jeffery, P. K. 2001. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 164(10 Pt 2):S28-38.

10. Holgate, S. T., P. Lackie, S. Wilson, W. Roche, and D. Davies. 2000. Bronchial epithelium as a key regulator of airway allergen sensitization and remodeling in asthma. *Am J Respir Crit Care Med* 162(3 Pt 2):S113-7.

11. Ten Hacken, N. H., D. S. Postma, and W. Timens. 2003. Airway remodeling and long-term decline in lung function in asthma. *Curr Opin Pulm Med* 9(1):9-14.

12. Kips, J. C., and R. A. Pauwels. 1999. Airway wall remodelling: does it occur and what does it mean? *Clin Exp Allergy* 29(11):1457-66.

13. Vignola, A. M., R. Gagliardo, A. Siena, G. Chiappara, M. R. Bonsignore, J. Bousquet, and G. Bonsignore. 2001. Airway remodeling in the pathogenesis of asthma. *Curr Allergy Asthma Rep* 1(2):108-15.

14. Davies, D. E., J. Wicks, R. M. Powell, S. M. Puddicombe, and S. T. Holgate. 2003. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 111(2):215-25; quiz 226.

15. Beckett, P. A., and P. H. Howarth. 2003. Pharmacotherapy and airway remodelling in asthma? *Thorax* 58(2):163-74.

16. Jansen, D. F., W. Timens, J. Kraan, B. Rijcken, and D. S. Postma. 1997. (A)symptomatic bronchial hyper-responsiveness and asthma. *Respir Med* 91(3):121-34.

17. Tashkin, D. P., M. D. Altose, E. R. Bleecker, J. E. Connett, R. E. Kanner, W. W. Lee, and R. Wise. 1992. The lung health study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. The Lung Health Study Research Group. *Am Rev Respir Dis* 145(2 Pt 1):301-10.

18. Van Asperen, P., C. M. Mellis, R. T. South, and S. J. Simpson. 1981. Bronchial reactivity in cystic fibrosis with normal pulmonary function. *Am J Dis Child* 135(9):815-9.

19. Pang, J., H. S. Chan, and J. Y. Sung. 1989. Prevalence of asthma, atopy, and bronchial hyperreactivity in bronchiectasis: a controlled study. *Thorax* 44(11):948-51.

20. Potena, A., R. La Corte, L. M. Fabbri, A. Papi, F. Trotta, and A. Ciaccia. 1990. Increased bronchial responsiveness in primary and secondary Sjogren's syndrome. *Eur Respir J* 3(5):548-53.

21. Alexander, H., and R. Paddock. 1921. Bronchial asthma: response to pilocarpine and epinepherine. *Arch Intern Med* 27:184-191.

22. Weiss, S., G. Robb, and L. Ellis. 1932. The systematic effects of hostamine in man. *Arch Intern Med* 49:360-396.

23. Tiffeneau, R., and R. Beauvallet. 1945. Epreuve de bronchoconstriction et de broncholilation par aerosols. *Bull Acad Med* 129:165-168.

24. Curry, J. 1946. The action of histamine on the respiratory tract of normal and asthmatic subjects. *J Clin Invest* 25:785-791.

25. O'Byrne, P. M., and M. D. Inman. 2003. Airway hyperresponsiveness. *Chest* 123(3 Suppl):411S-6S.

26. Cockcroft, D. W., D. N. Killian, J. J. Mellon, and F. E. Hargreave. 1977. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 7(3):235-43.

27. Chai, H., R. S. Farr, L. A. Froehlich, D. A. Mathison, J. A. McLean, R. R. Rosenthal, A. L. Sheffer, S. L. Spector, and R. G. Townley. 1975. Standardization of bronchial inhalation challenge procedures. *J Allergy Clin Immunol* 56(4):323-7.

28. Cockcroft, D. W., R. E. Ruffin, J. Dolovich, and F. E. Hargreave. 1977. Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 7(6):503-13.

29. Eiser, N., K. F. Kerrebijn, and P. H. Quanjer. 1983. Guidelines for standardization of bronchial challenges with (nonspecific) bronchoconstricting agents. *Bull Eur Physiopathol Respir* 19(5):495-514.

30. Sterk, P. J., L. M. Fabbri, P. H. Quanjer, D. W. Cockcroft, P. M. O'Byrne, S. D. Anderson, E. F. Juniper, and J. L. Malo. 1993. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 16:53-83.

31. Chinn, S. 1998. Methodology of bronchial responsiveness. *Thorax* 53(11):984-8.

32. Rubinfeld, A. R., and M. C. Pain. 1977. Relationship between bronchial reactivity, airway caliber, and severity of asthma. *Am Rev Respir Dis* 115(3):381-7.

33. Orehek, J., P. Gayrard, A. P. Smith, C. Grimaud, and J. Charpin. 1977. Airway response to carbachol in normal and asthmatic subjects: distinction between bronchial sensitivity and reactivity. *Am Rev Respir Dis* 115(6):937-43.

34. Yan, K., C. Salome, and A. J. Woolcock. 1983. Rapid method for measurement of bronchial responsiveness. *Thorax* 38(10):760-5.

35. Britton, J., A. Mortagy, and A. Tattersfield. 1986. Histamine challenge testing: comparison of three methods. *Thorax* 41(2):128-32.

36. Juniper, E. F., P. A. Frith, C. Dunnett, D. W. Cockcroft, and F. E. Hargreave. 1978. Reproducibility and comparison of responses to inhaled histamine and methacholine. *Thorax* 33(6):705-10.

37. Cockcroft, D. W., B. A. Berscheid, and K. Y. Murdock. 1983. Unimodal distribution of bronchial responsiveness to inhaled histamine in a random human population. *Chest* 83(5):751-4.

38. Josephs, L. K., I. Gregg, and S. T. Holgate. 1990. Does non-specific bronchial responsiveness indicate the severity of asthma? *Eur Respir J* 3(2):220-7.

39. Cockcroft, D. W., K. Y. Murdock, B. A. Berscheid, and B. P. Gore. 1992. Sensitivity and specificity of histamine PC20 determination in a random selection of young college students. *J Allergy Clin Immunol* 89(1 Pt 1):23-30.

40. Laprise, C., and L. P. Boulet. 1997. Asymptomatic airway hyperresponsiveness: a three-year follow-up. *Am J Respir Crit Care Med* 156(2 Pt 1):403-9.

41. Juniper, E. F., P. A. Frith, and F. E. Hargreave. 1981. Airway responsiveness to histamine and methacholine: relationship to minimum treatment to control symptoms of asthma. *Thorax* 36(8):575-9.

42. Sterk, P. J., M. C. Timmers, and J. H. Dijkman. 1986. Maximal airway narrowing in humans in vivo. Histamine compared with methacholine. *Am Rev Respir Dis* 134(4):714-8.

43. James, A., D. Lougheed, G. Pearce-Pinto, G. Ryan, and B. Musk. 1992. Maximal airway narrowing in a general population. *Am Rev Respir Dis* 146(4):895-9.

44. Longo, G., R. Strinati, F. Poli, and F. Fumi. 1987. Genetic factors in nonspecific bronchial hyperreactivity. An epidemiologic study. *Am J Dis Child* 141(3):331-4.

45. Hopp, R. J., A. K. Bewtra, R. Biven, N. M. Nair, and R. G. Townley. 1988. Bronchial reactivity pattern in nonasthmatic parents of asthmatics. *Ann Allergy* 61(3):184-6.

46. Nieminen, M. M., J. Kaprio, and M. Koskenvuo. 1991. A population-based study of bronchial asthma in adult twin pairs. *Chest* 100(1):70-5.

47. Duffy, D. L., N. G. Martin, D. Battistutta, J. L. Hopper, and J. D. Mathews. 1990. Genetics of asthma and hay fever in Australian twins. *Am Rev Respir Dis* 142(6 Pt 1):1351-8.

48. Postma, D. S., E. R. Bleecker, P. J. Amelung, K. J. Holroyd, J. Xu, C. I. Panhuysen, D. A. Meyers, and R. C. Levitt. 1995. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. *N Engl J Med* 333(14):894-900.

49. van Herwerden, L., S. B. Harrap, Z. Y. Wong, M. J. Abramson, J. J. Kutin, A. B. Forbes, J. Raven, A. Lanigan, and E. H. Walters. 1995. Linkage of high-affinity IgE receptor gene with bronchial hyperreactivity, even in absence of atopy. *Lancet* 346(8985):1262-5.

50. Van Eerdewegh, P., R. D. Little, J. Dupuis, R. G. Del Mastro, K. Falls, J. Simon, D. Torrey, S. Pandit, J. McKenny, K. Braunschweiger, A. Walsh, Z. Liu, B. Hayward, C. Folz, S. P. Manning, A. Bawa, L. Saracino, M. Thackston, Y. Benchekroun, N. Capparell, M. Wang, R. Adair, Y. Feng, J. Dubois, M. G. FitzGerald, H. Huang, R. Gibson, K. M. Allen, A. Pedan, M. R. Danzig, S. P. Umland, R. W. Egan, F. M. Cuss, S. Rorke, J. B. Clough, J. W. Holloway, S. T. Holgate, and T. P. Keith. 2002. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature*.

51. Bramley, A. M., M. N. Samhoun, and P. J. Piper. 1990. The role of the epithelium in modulating the responses of guinea-pig trachea induced by bradykinin in vitro. *Br J Pharmacol* 99(4):762-6.

52. Bousquet, J., P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, and et al. 1990. Eosinophilic inflammation in asthma. *N Engl J Med* 323(15):1033-9.

53. Ollerenshaw, S. L., and A. J. Woolcock. 1992. Characteristics of the inflammation in biopsies from large airways of subjects with asthma and subjects with chronic airflow limitation. *Am Rev Respir Dis* 145(4 Pt 1):922-7.

54. Djukanovic, R., J. W. Wilson, K. M. Britten, S. J. Wilson, A. F. Walls, W. R. Roche, P. H. Howarth, and S. T. Holgate. 1990. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 142(4):863-71.

55. Laitinen, L. A., A. Laitinen, and T. Haahtela. 1993. Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am Rev Respir Dis* 147(3):697-704.

56. Laitinen, L. A., A. Laitinen, A. Altraja, I. Virtanen, M. Kampe, B. G. Simonsson, S. E. Karlsson, L. Hakansson, P. Venge, and H. Sillastu. 1996. Bronchial biopsy findings in intermittent or "early" asthma. *J Allergy Clin Immunol* 98(5 Pt 2):S3-6; discussion S33-40.

57. Juniper, E. F., P. A. Kline, M. A. Vanzieleghem, E. H. Ramsdale, P. M. O'Byrne, and F. E. Hargreave. 1990. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. *Am Rev Respir Dis* 142(4):832-6.

58. Djukanovic, R., J. W. Wilson, K. M. Britten, S. J. Wilson, A. F. Walls, W. R. Roche, P. H. Howarth, and S. T. Holgate. 1992. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 145(3):669-74.

59. Djukanovic, R., S. Homeyard, C. Gratziou, J. Madden, A. Walls, S. Montefort, D. Peroni, R. Polosa, S. Holgate, and P. Howarth. 1997. The effect of treatment with oral corticosteroids on asthma symptoms and airway inflammation. *Am J Respir Crit Care Med* 155(3):826-32.

60. Haahtela, T., M. Jarvinen, T. Kava, K. Kiviranta, S. Koskinen, K. Lehtonen, K. Nikander, T. Persson, K. Reinikainen, O. Selroos, and et al. 1991. Comparison of a beta 2-agonist, terbutaline, with an inhaled corticosteroid, budesonide, in newly detected asthma. *N Engl J Med* 325(6):388-92.

61. van Essen-Zandvliet, E. E., M. D. Hughes, H. J. Waalkens, E. J. Duiverman, S. J. Pocock, and K. F. Kerrebijn. 1992. Effects of 22 months of treatment with inhaled corticosteroids and/or beta-2-agonists on lung function, airway responsiveness, and symptoms in children with asthma. The Dutch Chronic Non-specific Lung Disease Study Group. *Am Rev Respir Dis* 146(3):547-54.

62. British Thoracic Society. 2003. British guideline on the management of asthma. *Thorax* 58 Suppl 1:i1-94.

63. Holgate, S. T. 2000. Airway wall remodelling. *Eur Respir Rev* 10(69):58-63.

64. Brown, P. J., H. W. Greville, and K. E. Finucane. 1984. Asthma and irreversible airflow obstruction. *Thorax* 39(2):131-6.

65. Laitinen, L. A., A. Laitinen, and T. Haahtela. 1992. A comparative study of the effects of an inhaled corticosteroid, budesonide, and a beta 2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: a randomized, double-blind, parallel-group controlled trial. *J Allergy Clin Immunol* 90(1):32-42.

66. Selroos, O., A. Pietinalho, A. B. Lofroos, and H. Riska. 1995. Effect of early vs late intervention with inhaled corticosteroids in asthma. *Chest* 108(5):1228-34.

67. Boulet, L. P., H. Turcotte, M. Laviolette, F. Naud, M. C. Bernier, S. Martel, and J. Chakir. 2000. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* 162(4 Pt 1):1308-13.

68. Dunnill, M. S., G. R. Massarella, and J. A. Anderson. 1969. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24(2):176-9.

69. Aikawa, T., S. Shimura, H. Sasaki, M. Ebina, and T. Takishima. 1992. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 101(4):916-21.

70. Roche, W. R., R. Beasley, J. H. Williams, and S. T. Holgate. 1989. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1(8637):520-4.

71. Cho, S. H., J. Y. Seo, D. C. Choi, H. J. Yoon, Y. J. Cho, K. U. Min, G. K. Lee, J. W. Seo, and Y. Y. Kim. 1996. Pathological changes according to the severity of asthma. *Clin Exp Allergy* 26(10):1210-9.

72. James, A. L., P. D. Pare, and J. C. Hogg. 1989. The mechanics of airway narrowing in asthma. *Am Rev Respir Dis* 139(1):242-6.

73. Heard, B. E., Hossain, S. 1973. Hyperplasia of bronchial smooth muscle in asthma. *J Pathol* 110:319-331.

74. Ebina, M., T. Takahashi, T. Chiba, and M. Motomiya. 1993. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am Rev Respir Dis* 148(3):720-6.

75. Carroll, N., J. Elliot, A. Morton, and A. James. 1993. The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis* 147(2):405-10.

76. Huber, H. L., Kossler, K.K. 1922. The pathology of bronchial asthma. *Arch Intern Med* 30:189-760.

77. Kuwano, K., C. H. Bosken, P. D. Pare, T. R. Bai, B. R. Wiggs, and J. C. Hogg. 1993. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 148(5):1220-5.

78. Djukanovic, R., W. R. Roche, J. W. Wilson, C. R. Beasley, O. P. Twentyman, R. H. Howarth, and S. T. Holgate. 1990. Mucosal inflammation in asthma. *Am Rev Respir Dis* 142(2):434-57.

79. Balzar, S., S. E. Wenzel, and H. W. Chu. 2002. Transbronchial biopsy as a tool to evaluate small airways in asthma. *Eur Respir J* 20:254-259.

80. Laprise, C., M. Laviolette, M. Boutet, and L. P. Boulet. 1999. Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling. *Eur Respir J* 14(1):63-73.

81. Houston, J. C., S. de Navasquez, and J. R. Trounce. 1953. A Clinical and pathological study of fatal cases of status asthmaticus. *Thorax* 8:207-213.

82. Messer, J. W., G. A. Peters, W. A. Bennett, and J. R. Trounce. 1960. Causes of death and pathological findings in 304 cases of bronchial asthma. *Dis Chest* 38:616-624.

83. Dunnill, M. S. 1960. The pathology of asthma, with special reference to changes in the bronchial mucosa. *J Clin Pathol* 13:27-33.

84. James, A. L., P. D. Pare, R. H. Moreno, and J. C. Hogg. 1987. Quantitative measurement of smooth muscle shortening in isolated pig trachea. *J Appl Physiol* 63(4):1360-5.

85. James, A. L., J. C. Hogg, L. A. Dunn, and P. D. Pare. 1988. The use of the internal perimeter to compare airway size and to calculate smooth muscle shortening. *Am Rev Respir Dis* 138(1):136-9.

86. Bai, T. R., J. Cooper, T. Koelmeyer, P. D. Pare, and T. D. Weir. 2000. The effect of age and duration of disease on airway structure in fatal asthma. *Am J Respir Crit Care Med* 162(2 Pt 1):663-9.

87. Montefort, S., J. A. Roberts, R. Beasley, S. T. Holgate, and W. R. Roche. 1992. The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects. *Thorax* 47(7):499-503.

88. Lozewicz, S., C. Wells, E. Gomez, H. Ferguson, P. Richman, J. Devalia, and R. J. Davies. 1990. Morphological integrity of the bronchial epithelium in mild asthma. *Thorax* 45(1):12-5.

89. Jeffery, P. K., A. J. Wardlaw, F. C. Nelson, J. V. Collins, and A. B. Kay. 1989. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 140(6):1745-53.

90. Boulet, L. P., M. Laviolette, H. Turcotte, A. Cartier, M. Dugas, J. L. Malo, and M. Boutet. 1997. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* 112(1):45-52.

91. Huber, H. L., and K. K. Koessler. 1922. The pathology of bronchial asthma. *Arch Internal Medicine* 30:689-760.

92. Salvato, G. 1968. Some histological changes in chronic bronchitis and asthma. *Thorax* 23(2):168-72.

93. Cutz, E., H. Levison, and D. M. Cooper. 1978. Ultrastructure of airways in children with asthma. *Histopathology* 2(6):407-21.

94. Cokugras, H., N. Akcakaya, Seckin, Y. Camcioglu, N. Sarimurat, and F. Aksoy. 2001. Ultrastructural examination of bronchial biopsy specimens from children with moderate asthma. *Thorax* 56(1):25-9.

95. Chakir, J., M. Laviolette, M. Boutet, R. Laliberte, J. Dube, and L. P. Boulet. 1996. Lower airways remodeling in nonasthmatic subjects with allergic rhinitis. *Lab Invest* 75(5):735-44.

96. Brewster, C. E., P. H. Howarth, R. Djukanovic, J. Wilson, S. T. Holgate, and W. R. Roche. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 3(5):507-11.

97. Chetta, A., A. Foresi, M. Del Donno, G. Bertorelli, A. Pesci, and D. Olivieri. 1997. Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 111(4):852-7.

98. Laitinen, A., A. Altraja, M. Kampe, M. Linden, I. Virtanen, and L. A. Laitinen. 1997. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 156(3 Pt 1):951-8.

99. Altraja, A., A. Laitinen, I. Virtanen, M. Kampe, B. G. Simonsson, S. E. Karlsson, L. Hakansson, P. Venge, H. Sillastu, and L. A. Laitinen. 1996. Expression of laminins in the airways in various types of asthmatic patients: a morphometric study. *Am J Respir Cell Mol Biol* 15(4):482-8.

100. Wilson, J. W., and X. Li. 1997. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy* 27(4):363-71.

101. Chu, H. W., J. L. Halliday, R. J. Martin, D. Y. Leung, S. J. Szefler, and S. E. Wenzel. 1998. Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease. *Am J Respir Crit Care Med* 158(6):1936-44.

102. Saetta, M., A. Di Stefano, C. Rosina, G. Thiene, and L. M. Fabbri. 1991. Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *Am Rev Respir Dis* 143(1):138-43.

103. Brusasco, V., E. Crimi, and R. Pellegrino. 1998. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* 53(11):992-8.

104. Mitchell, R. W., E. Ruhlmann, H. Magnussen, A. R. Leff, and K. F. Rabe. 1994. Passive sensitization of human bronchi augments smooth muscle shortening velocity and capacity. *Am J Physiol* 267(2 Pt 1):L218-22.

105. Solway, J., and J. J. Fredberg. 1997. Perhaps airway smooth muscle dysfunction contributes to asthmatic bronchial hyperresponsiveness after all. *Am J Respir Cell Mol Biol* 17(2):144-6.

106. Carroll, N. G., C. Cooke, and A. L. James. 1997. Bronchial blood vessel dimensions in asthma. *Am J Respir Crit Care Med* 155(2):689-95.

107. Li, X., and J. W. Wilson. 1997. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med* 156(1):229-33.

108. Orsida, B. E., C. Ward, X. Li, R. Bish, J. W. Wilson, F. Thien, and E. H. Walters. 2001. Effect of a long-acting beta2-agonist over three months on airway wall vascular remodeling in asthma. *Am J Respir Crit Care Med* 164(1):117-21.

109. Vrugt, B., S. Wilson, A. Bron, S. T. Holgate, R. Djukanovic, and R. Aalbers. 2000. Bronchial angiogenesis in severe glucocorticoid-dependent asthma. *Eur Respir J* 15(6):1014-21.

110. Godfrey, R. W., S. Lorimer, S. Majumdar, E. Adelroth, P. W. Johnston, A. V. Rogers, S. A. Johansson, and P. K. Jeffery. 1995. Airway and lung elastic fibre is not reduced in asthma nor in asthmatics following corticosteroid treatment. *Eur Respir J* 8(6):922-7.

111. Bousquet, J., J. Y. Lacoste, P. Chanez, P. Vic, P. Godard, and F. B. Michel. 1996. Bronchial elastic fibers in normal subjects and asthmatic patients. *Am J Respir Crit Care Med* 153(5):1648-54.

112. Carroll, N. G., S. Perry, A. Karkhanis, S. Harji, J. Butt, A. L. James, and F. H. Y. Green. 2000. The Airway Longitudinal Elastic Fiber Network and Mucosal Folding in Patients with Asthma. *Am. J. Respir. Crit. Care Med.* 161(1):244-248.

113. Westergren-Thorsson, G., J. Chakir, M. J. Lafreniere-Allard, L. P. Boulet, and G. M. Tremblay. 2002. Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects. *Int J Biochem Cell Biol* 34(10):1256-67.

114. Huang, J., R. Olivenstein, R. Taha, Q. Hamid, and M. Ludwig. 1999. Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. *Am J Respir Crit Care Med* 160(2):725-9.

115. Redington, A. E., W. R. Roche, S. T. Holgate, and P. H. Howarth. 1998. Co-localization of immunoreactive transforming growth factor-beta 1 and decorin in bronchial biopsies from asthmatic and normal subjects. *J Pathol* 186(4):410-5.

116. Holgate, S. T., D. E. Davies, P. M. Lackie, S. J. Wilson, S. M. Puddicombe, and J. L. Lordan. 2000. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 105(2 Pt 1):193-204.

117. Ordonez, C. L., R. Khashayar, H. H. Wong, R. Ferrando, R. Wu, D. M. Hyde, J. A. Hotchkiss, Y. Zhang, A. Novikov, G. Dolganov, and J. V. Fahy. 2001. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* 163(2):517-23.

118. Holgate, S. T. 2000. Epithelial damage and response. *Clin Exp Allergy* 30 Suppl 1:37-41.

119. Puddicombe, S. M., R. Polosa, A. Richter, M. T. Krishna, P. H. Howarth, S. T. Holgate, and D. E. Davies. 2000. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *Faseb J* 14(10):1362-74.

120. Lordan, J. L., F. Bucchieri, A. Richter, A. Konstantinidis, J. W. Holloway, M. Thornber, S. M. Puddicombe, D. Buchanan, S. J. Wilson, R. Djukanovic, S. T. Holgate, and D. E. Davies. 2002. Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. *J Immunol* 169(1):407-14.

121. Kumar, R. K., C. Herbert, M. Yang, A. M. Koskinen, A. N. McKenzie, and P. S. Foster. 2002. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. *Clin Exp Allergy* 32(7):1104-11.

122. Burgel, P. R., S. C. Lazarus, D. C. Tam, I. F. Ueki, K. Atabai, M. Birch, and J. A. Nadel. 2001. Human eosinophils induce mucin production in airway epithelial cells via epidermal growth factor receptor activation. *J Immunol* 167(10):5948-54.

123. Richter, A., S. M. Puddicombe, J. L. Lordan, F. Bucchieri, S. J. Wilson, R. Djukanovic, G. Dent, S. T. Holgate, and D. E. Davies. 2001. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol Biol* 25(3):385-91.

124. Moreno, R. H., J. C. Hogg, and P. D. Pare. 1986. Mechanics of airway narrowing. *Am Rev Respir Dis* 133(6):1171-80.

125. Weibel, E. R. 1963. Morphometry of the human lung. Springer, Berlin.

126. Wiggs, B. R., R. Moreno, J. C. Hogg, C. Hilliam, and P. D. Pare. 1990. A model of the mechanics of airway narrowing. *J Appl Physiol* 69(3):849-60.

127. Okazawa, M., P. Pare, and J. Road. 1990. Tracheal smooth muscle mechanics in vivo. *J Appl Physiol* 68(1):209-19.

128. Lambert, R. K., B. R. Wiggs, K. Kuwano, J. C. Hogg, and P. D. Pare. 1993. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 74(6):2771-81.

129. Wiggs, B. R., C. A. Hrousis, J. M. Drazen, and R. D. Kamm. 1997. On the mechanism of mucosal folding in normal and asthmatic airways. *J Appl Physiol* 83(6):1814-21.

130. Lum, H., and W. Mitzner. 1985. Effects of 10% formalin fixation on fixed lung volume and lung tissue shrinkage. A comparison of eleven laboratory species. *Am Rev Respir Dis* 132(5):1078-83.

131. Minshall, E. M., J. C. Hogg, and Q. A. Hamid. 1998. Cytokine mRNA expression in asthma is not restricted to the large airways. *J Allergy Clin Immunol* 101(3):386-90.

132. Hamid, Q., Y. Song, T. C. Kotsimbos, E. Minshall, T. R. Bai, R. G. Hegele, and J. C. Hogg. 1997. Inflammation of small airways in asthma. *J Allergy Clin Immunol* 100(1):44-51.

133. Lynch, D. A., J. D. Newell, B. A. Tschomper, T. M. Cink, L. S. Newman, and R. Bethel. 1993. Uncomplicated asthma in adults: comparison of CT appearance of the lungs in asthmatic and healthy subjects. *Radiology* 188(3):829-33.

134. Paganin, F., E. Seneterre, P. Chanez, J. P. Daures, J. M. Bruel, F. B. Michel, and J. Bousquet. 1996. Computed tomography of the lungs in asthma: influence of disease severity and etiology. *Am J Respir Crit Care Med* 153(1):110-4.

135. Boulet, L., M. Belanger, and G. Carrier. 1995. Airway responsiveness and bronchial-wall thickness in asthma with or without fixed airflow obstruction. *Am J Respir Crit Care Med* 152(3):865-71.

136. Okazawa, M., N. Muller, A. E. McNamara, S. Child, L. Verburgt, and P. D. Pare. 1996. Human airway narrowing measured using high resolution computed tomography. *Am J Respir Crit Care Med* 154(5):1557-62.

137. Park, J. W., Y. K. Hong, C. W. Kim, D. K. Kim, K. O. Choe, and C. S. Hong. 1997. High-resolution computed tomography in patients with bronchial asthma: correlation with clinical features, pulmonary functions and bronchial hyperresponsiveness. *J Investig Allergol Clin Immunol* 7(3):186-92.

138. Awadh, N., N. L. Muller, C. S. Park, R. T. Abboud, and J. M. FitzGerald. 1998. Airway wall thickness in patients with near fatal asthma and control groups: assessment with high resolution computed tomographic scanning. *Thorax* 53(4):248-53.

139. Niimi, A., H. Matsumoto, R. Amitani, Y. Nakano, M. Mishima, M. Minakuchi, K. Nishimura, H. Itoh, and T. Izumi. 2000. Airway wall thickness in asthma assessed by computed tomography. Relation to clinical indices. *Am J Respir Crit Care Med* 162(4 Pt 1):1518-23.

140. Kasahara, K., K. Shiba, T. Ozawa, K. Okuda, and M. Adachi. 2002. Correlation between the bronchial subepithelial layer and whole airway wall thickness in patients with asthma. *Thorax* 57(3):242-6.

141. Niimi, A., H. Matsumoto, M. Takemura, T. Ueda, K. Chin, and M. Mishima. 2003. Relationship of airway wall thickness to airway sensitivity and airway reactivity in asthma. *Am J Respir Crit Care Med* 168(8):983-8.

142. Little, S. A., M. W. Sproule, M. D. Cowan, K. J. Macleod, M. Robertson, J. G. Love, G. W. Chalmers, C. P. McSharry, and N. C. Thomson. 2002. High resolution computed tomographic assessment of airway wall thickness in chronic asthma: reproducibility and relationship with lung function and severity. *Thorax* 57(3):247-53.

143. King, G. G., N. L. Muller, K. P. Whittall, Q. S. Xiang, and P. D. Pare. 2000. An analysis algorithm for measuring airway lumen and wall areas from high-resolution computed tomographic data. *Am J Respir Crit Care Med* 161(2 Pt 1):574-80.

144. McNamara, A. E., N. L. Muller, M. Okazawa, J. Arntorp, B. R. Wiggs, and P. D. Pare. 1992. Airway narrowing in excised canine lungs measured by high-resolution computed tomography. *J Appl Physiol* 73(1):307-16.

145. King, G. G., N. L. Muller, and P. D. Pare. 1999. Evaluation of airways in obstructive pulmonary disease using high-resolution computed tomography. *Am J Respir Crit Care Med* 159(3):992-1004.

146. Bland, J. M., and D. G. Altman. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1(8476):307-10.

147. Nakano, Y., S. Muro, H. Sakai, T. Hirai, K. Chin, M. Tsukino, K. Nishimura, H. Itoh, P. D. Pare, J. C. Hogg, and M. Mishima. 2000. Computed tomographic measurements of airway dimensions and emphysema in smokers. Correlation with lung function. *Am J Respir Crit Care Med* 162(3 Pt 1):1102-8.

148. Wood, S. A., E. A. Zerhouni, J. D. Hoford, E. A. Hoffman, and W. Mitzner. 1995. Measurement of three-dimensional lung tree structures by using computed tomography. *J Appl Physiol* 79(5):1687-97.

149. Boiselle, P. M., and A. Ernst. 2002. Recent advances in central airway imaging. *Chest* 121(5):1651-60.

150. European Commission. 2000. Referral guidelines for imaging Radiation Protection 118.

151. ImPACT. 2002. http://www.impactscan.org. Version 0.99m ed. Medical Physics Department at St George's Hospital, London.

152. Jones, D., and P. Shrimpton. 1991. Report SR250, Normalised Organ Doses for X-Ray Computed Tomography Calculated Using Monte Carlo Techniques. National Radiological Protection Board, Didcot, Oxon, UK.

153. Silverstein, F. E., R. W. Martin, M. B. Kimmey, G. C. Jiranek, D. W. Franklin, and A. Proctor. 1989. Experimental evaluation of an endoscopic ultrasound probe: in vitro and in vivo canine studies. *Gastroenterology* 96(4):1058-62.

154. Botet, J. F., C. J. Lightdale, A. G. Zauber, H. Gerdes, C. Urmacher, and M. F. Brennan. 1991. Preoperative staging of esophageal cancer: comparison of endoscopic US and dynamic CT. *Radiology* 181(2):419-25.

155. Caletti, G., and P. Fusaroli. 1999. Endoscopic ultrasonography. *Endoscopy* 31(1):95-102.

156. Rodes-Cabau, J., E. Domingo, A. Roman, J. Majo, B. Lara, F. Padilla, I. Anivarro, J. Angel, J. C. Tardif, and J. Soler-Soler. 2003. Intravascular ultrasound of the elastic pulmonary arteries: a new approach for the evaluation of primary pulmonary hypertension. *Heart* 89(3):311-5.

157. Hoffmann, K., M. Happe, S. Schuller, M. Stucker, M. Wiesner, P. Gottlober, M. Schwarz, J. Strahler, H. Neubauer, C. Jung, S. Petereit, J. Welzel, N. Brautzsch, J. Bohmeyer, J. Wohlrab, M. Freitag, and P. Altmeyer. 1999. [Ranking of 20 MHz sonography of malignant melanoma and pigmented lesions in routine diagnosis]. *Ultraschall Med* 20(3):104-9.

158. Hurter, T., and P. Hanrath. 1992. Endobronchial sonography: feasibility and preliminary results. *Thorax* 47(7):565-7.

159. Burgers, J. A., F. Herth, and H. D. Becker. 2001. Endobronchial ultrasound. *Lung Cancer* 34 Suppl 2:S109-13.

160. Kurimoto, N., M. Murayama, S. Yoshioka, T. Nishisaka, K. Inai, and K. Dohi. 1999. Assessment of usefulness of endobronchial ultrasonography in determination of depth of tracheobronchial tumor invasion. *Chest* 115(6):1500-6.

161. Tanaka, F., K. Muro, S. Yamasaki, G. Watanabe, Y. Shimada, M. Imamura, S. Hitomi, and H. Wada. 2000. Evaluation of tracheo-bronchial wall invasion using transbronchial ultrasonography (TBUS). *Eur J Cardiothorac Surg* 17(5):570-4.

162. Baba, M., Y. Sekine, M. Suzuki, S. Yoshida, K. Shibuya, T. Iizasa, Y. Saitoh, E. K. Onuma, H. Ohwada, and T. Fujisawa. 2002. Correlation between endobronchial ultrasonography (EBUS) images and histologic findings in normal and tumor-invaded bronchial wall. *Lung Cancer* 35(1):65-71.

163. Djukanovic, R., R. Dahl, N. Jarjour, and R. Aalbers. 1998. Safety of biopsies and bronchoalveolar lavage. *Eur Respir J Suppl* 26:39S-41S.

164. Djukanovic, R., J. W. Wilson, C. K. Lai, S. T. Holgate, and P. H. Howarth. 1991. The safety aspects of fiberoptic bronchoscopy, bronchoalveolar lavage, and endobronchial biopsy in asthma. *Am Rev Respir Dis* 143(4 Pt 1):772-7.

165. British Thoracic Society. 2001. British Thoracic Society guidelines on diagnostic flexible bronchoscopy. *Thorax* 56 Suppl 1:i1-21.

166. University of Geneva. 2003. Osiris. 4.18 ed.

167. Britten, K. M., P. H. Howarth, and W. R. Roche. 1993. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. *Biotech Histochem* 68(5):271-80.

168. Sullivan, P., D. Stephens, T. Ansari, J. Costello, and P. Jeffery. 1998. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 12(4):811-5.

169. Herth, F., A. Ernst, M. Schulz, and H. Becker. 2003. Endobronchial ultrasound reliably differentiates between airway infiltration and compression by tumor. *Chest* 123(2):458-62.

170. Kurimoto, N., M. Murayama, S. Yoshioka, and T. Nishisaka. 2002. Analysis of the internal structure of peripheral pulmonary lesions using endobronchial ultrasonography. *Chest* 122(6):1887-94.

171. Iwadare, T., H. Mori, K. Ishiguro, and M. Takeishi. 1984. Dimensional changes of tissues in the course of processing. *J Microsc* 136 (Pt 3):323-7.

172. Kimura, M., N. Tayama, and R. W. Chan. 2003. Geometrical deformation of vocal fold tissues induced by formalin fixation. *Laryngoscope* 113(4):607-13.

173. Becker, H. D. 1996. [Endobronchial ultrasound--a new perspective in bronchology]. *Ultraschall Med* 17(3):106-12.

174. Jeffery, P. 2002. Microscopic structure of the lung. *In* G. Gibson, D. Geddes, U. Costabel, P. Sterk and B. Corrin, editors. Respiratory Medicine. Saunders. 34-50.

175. Kurimoto, N., M. Murayama, K. Morita, A. Kabayashi, M. Uomoto, and T. Nishizaka. 1998. Clinical applications of endobronchial ultrasound in lung disease. *Endoscopy* 30(Suppl. 1):A8-A12.

176. Saisho, H., K. Sai, T. Tsuyuguchi, T. Yamaguchi, S. Matsutani, and M. Ohto. 1995. A new small probe for ultrasound imaging via conventional endoscope. *Gastrointest Endosc* 41(2):141-5.

177. Tamada, K., K. Ido, N. Ueno, K. Kimura, M. Ichiyama, and T. Tomiyama. 1995. Preoperative staging of extrahepatic bile duct cancer with intraductal ultrasonography. *Am J Gastroenterol* 90(2):239-46.

178. Irani, S., T. Hess, M. Hofer, A. Gaspert, L. M. Bachmann, E. W. Russi, and A. Boehler. 2006. Endobronchial ultrasonography for the quantitative assessment of bronchial mural structures in lung transplant recipients. *Chest* 129(2):349-55.

179. Gono, H., K. Fujimoto, S. Kawakami, and K. Kubo. 2003. Evaluation of airway wall thickness and air trapping by HRCT in asymptomatic asthma. *Eur Respir J* 22(6):965-71.

180. McLean, A. N., M. W. Sproule, M. D. Cowan, and N. C. Thomson. 1998. High resolution computed tomography in asthma. *Thorax* 53(4):308-14.

181. Webb, W. R., G. Gamsu, S. D. Wall, C. E. Cann, and E. Proctor. 1984. CT of a bronchial phantom. Factors affecting appearance and size measurements. *Invest Radiol* 19(5):394-8.

182. Seneterre, E., F. Paganin, J. M. Bruel, F. B. Michel, and J. Bousquet. 1994. Measurement of the internal size of bronchi using high resolution computed tomography (HRCT). *Eur Respir J* 7(3):596-600.

183. Wiggs, B. R., C. Bosken, P. D. Pare, A. James, and J. C. Hogg. 1992. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 145(6):1251-8.

184. Pare, P. D., C. R. Roberts, T. R. Bai, and B. J. Wiggs. 1997. The functional consequences of airway remodeling in asthma. *Monaldi Arch Chest Dis* 52(6):589-96.

185. McParland, B. E., P. R. Johnson, C. L. Armour, and J. L. Black. 2001. Human bronchial internal perimeter is not constant. *Am J Respir Crit Care Med* 163:A415.

186. Okazawa, M., S. Vedal, L. Verburgt, R. K. Lambert, and P. D. Pare. 1995. Determinants of airway smooth muscle shortening in excised canine lobes. *J Appl Physiol* 78(2):608-14.

187. Moreno, R. H., J. C. Hogg, T. M. McLean, and P. D. Pare. 1987. Isovolumetric and isobaric rabbit tracheal contraction in vitro. *J Appl Physiol* 62(1):82-90.

188. Fabbri, L. M., G. Caramori, B. Beghe, A. Papi, and A. Ciaccia. 1998. Physiologic consequences of long-term inflammation. *Am J Respir Crit Care Med* 157(5 Pt 2):S195-8.

189. Holgate, S. T. 2000. Inflammatory and structural changes in the airways of patients with asthma. *Respir Med* 94 Suppl D:S3-6.

190. Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M. S. Ludwig, N. Barnes, D. Robinson, and A. B. Kay. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 112(7):1029-36.

191. Humbles, A. A., C. M. Lloyd, S. J. McMillan, D. S. Friend, G. Xanthou, E. E. McKenna, S. Ghiran, N. P. Gerard, C. Yu, S. H. Orkin, and C. Gerard. 2004. A Critical Role for Eosinophils in Allergic Airways Remodeling. *Science* 305(5691):1776-1779.

192. Lee, J. J., M. P. McGarry, S. C. Farmer, K. L. Denzler, K. A. Larson, P. E. Carrigan, I. E. Brenneise, M. A. Horton, A. Haczku, E. W. Gelfand, G. D. Leikauf, and N. A. Lee. 1997. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* 185(12):2143-56.

193. Brown, R. H., E. A. Zerhouni, and W. Mitzner. 1995. Airway edema potentiates airway reactivity. *J Appl Physiol* 79(4):1242-8.

194. Jeffery, P. K., R. W. Godfrey, E. Adelroth, F. Nelson, A. Rogers, and S. A. Johansson. 1992. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. *Am Rev Respir Dis* 145(4 Pt 1):890-9.

195. Ward, C., M. Pais, R. Bish, D. Reid, B. Feltis, D. Johns, and E. H. Walters. 2002. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 57(4):309-16.

196. American Thoracic Society. 1987. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. *Am Rev Respir Dis* 136(1):225-44.

197. Aas, K. 1981. Heterogeneity of bronchial asthma. Sub-populations--or different stages of the disease. *Allergy* 36(1):3-14.

198. Lim, S., A. Jatakanon, M. John, T. Gilbey, J. O'Connor B, K. F. Chung, and P. J. Barnes. 1999. Effect of inhaled budesonide on lung function and airway inflammation. Assessment by various inflammatory markers in mild asthma. *Am J Respir Crit Care Med* 159(1):22-30.

199. Lundgren, R., M. Soderberg, P. Horstedt, and R. Stenling. 1988. Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur Respir J* 1(10):883-9.

200. Moller, G. M., S. E. Overbeek, C. G. Van Helden-Meeuwsen, J. M. Van Haarst, E. P. Prens, P. G. Mulder, D. S. Postma, and H. C. Hoogsteden. 1996. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. *Clin Exp Allergy* 26(5):517-24.

201. Trigg, C. J., N. D. Manolitsas, J. Wang, M. A. Calderon, A. McAulay, S. E. Jordan, M. J. Herdman, N. Jhalli, J. M. Duddle, S. A. Hamilton, and et al. 1994. Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma. *Am J Respir Crit Care Med* 150(1):17-22.

202. Burke, C., C. K. Power, A. Norris, A. Condez, B. Schmekel, and L. W. Poulter. 1992. Lung function and immunopathological changes after inhaled corticosteroid therapy in asthma. *Eur Respir J* 5(1):73-9.

203. Burke, C. M., S. Sreenan, S. Pathmakanthan, J. Patterson, B. Schmekel, and L. W. Poulter. 1996. Relative effects of inhaled corticosteroids on immunopathology and physiology in asthma: a controlled study. *Thorax* 51(10):993-9.

204. Hoshino, M., Y. Nakamura, J. J. Sim, Y. Yamashiro, K. Uchida, K. Hosaka, and S. Isogai. 1998. Inhaled corticosteroid reduced lamina reticularis of the basement

membrane by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma. *Clin Exp Allergy* 28(5):568-77.

205. Hoshino, M., M. Takahashi, Y. Takai, and J. Sim. 1999. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 104(2 Pt 1):356-63.

206. Hoshino, M., M. Takahashi, Y. Takai, J. Sim, and N. Aoike. 2001. Inhaled corticosteroids decrease vascularity of the bronchial mucosa in patients with asthma. *Clinical & Experimental Allergy* 31(5):722-30.

207. Orsida, B. E., X. Li, B. Hickey, F. Thien, J. W. Wilson, and E. H. Walters. 1999. Vascularity in asthmatic airways: relation to inhaled steroid dose. *Thorax* 54(4):289-95.

208. Vanacker, N. J., E. Palmans, R. A. Pauwels, and J. C. Kips. 2002. Effect of combining salmeterol and fluticasone on the progression of airway remodeling. *Am J Respir Crit Care Med* 166(8):1128-34.

209. Vanacker, N. J., E. Palmans, R. A. Pauwels, and J. C. Kips. 2002. Fluticasone inhibits the progression of allergen-induced structural airway changes. *Clin Exp Allergy* 32(6):914-20.

210. Bradley, B. L., M. Azzawi, M. Jacobson, B. Assoufi, J. V. Collins, A. M. Irani, L. B. Schwartz, S. R. Durham, P. K. Jeffery, and A. B. Kay. 1991. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 88(4):661-74.

211. Kraft, M., R. Djukanovic, S. Wilson, S. T. Holgate, and R. J. Martin. 1996. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 154(5):1505-10.

212. Kraft, M., R. J. Martin, S. Wilson, R. Djukanovic, and S. T. Holgate. 1999. Lymphocyte and eosinophil influx into alveolar tissue in nocturnal asthma. *Am J Respir Crit Care Med* 159(1):228-34.

213. Duddridge, M., C. Ward, D. J. Hendrick, and E. H. Walters. 1993. Changes in bronchoalveolar lavage inflammatory cells in asthmatic patients treated with high dose inhaled beclomethasone dipropionate. *Eur Respir J* 6(4):489-97.

214. Olivieri, D., A. Chetta, M. Del Donno, G. Bertorelli, A. Casalini, A. Pesci, R. Testi, and A. Foresi. 1997. Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study. *Am J Respir Crit Care Med* 155(6):1864-71.

215. Wenzel, S. E., L. B. Schwartz, E. L. Langmack, J. L. Halliday, J. B. Trudeau, R. L. Gibbs, and H. W. Chu. 1999. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 160(3):1001-8.

216. Simpson, J. L., R. J. Scott, M. J. Boyle, and P. G. Gibson. 2005. Differential proteolytic enzyme activity in eosinophilic and neutrophilic asthma. *Am J Respir Crit Care Med* 172(5):559-65.

217. Ebina, M., H. Yaegashi, R. Chiba, T. Takahashi, M. Motomiya, and M. Tanemura. 1990. Hyperreactive site in the airway tree of asthmatic patients revealed by thickening of bronchial muscles. A morphometric study. *Am Rev Respir Dis* 141(5 Pt 1):1327-32.

218. Minshall, E., D. Leung, R. Martin, Y. Song, L. Cameron, P. Ernst, and Q. Hamid. 1997. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 17(3):326-333.

219. Chakir, J., J. Shannon, S. Molet, M. Fukakusa, J. Elias, M. Laviolette, L. P. Boulet, and Q. Hamid. 2003. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 111(6):1293-8.

220. Saglani, S., G. Papaioannou, L. Khoo, M. Ujita, P. K. Jeffery, C. Owens, D. M. Hansell, D. N. Payne, and A. Bush. 2006. Can HRCT be used as a marker of airway remodelling in children with difficult asthma? *Respir Res* 7:46.

221. Romberger, D. J., J. D. Beckmann, L. Claassen, R. F. Ertl, and S. I. Rennard. 1992. Modulation of fibronectin production of bovine bronchial epithelial cells by transforming growth factor-beta. *Am J Respir Cell Mol Biol* 7(2):149-55.

222. Harkonen, E., I. Virtanen, A. Linnala, L. L. Laitinen, and V. L. Kinnula. 1995. Modulation of fibronectin and tenascin production in human bronchial epithelial cells by inflammatory cytokines in vitro. *Am J Respir Cell Mol Biol* 13(1):109-15.

223. Palmans, E., J. C. Kips, and R. A. Pauwels. 2000. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 161(2 Pt 1):627-35.

224. Ruoslahti, E., and Y. Yamaguchi. 1991. Proteoglycans as modulators of growth factor activities. *Cell* 64(5):867-9.

225. Miyazono, K., H. Ichijo, and C. H. Heldin. 1993. Transforming growth factor-beta: latent forms, binding proteins and receptors. *Growth Factors* 8(1):11-22.

226. Redington, A. E., J. Madden, A. J. Frew, R. Djukanovic, W. R. Roche, S. T. Holgate, and P. H. Howarth. 1997. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 156(2 Pt 1):642-7.

227. Boxall, C., S. T. Holgate, and D. E. Davies. 2006. The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* 27(1):208-29.

228. Mautino, G., N. Oliver, P. Chanez, J. Bousquet, and F. Capony. 1997. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 17(5):583-91.

229. Kelly, E. A., W. W. Busse, and N. N. Jarjour. 2000. Increased matrix metalloproteinase-9 in the airway after allergen challenge. *Am J Respir Crit Care Med* 162(3 Pt 1):1157-61.

230. Vignola, A. M., L. Riccobono, A. Mirabella, M. Profita, P. Chanez, V. Bellia, G. Mautino, P. D'Accardi, J. Bousquet, and G. Bonsignore. 1998. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 158(6):1945-50.

231. Matsumoto, H., A. Niimi, M. Takemura, T. Ueda, M. Minakuchi, R. Tabuena, K. Chin, T. Mio, Y. Ito, S. Muro, T. Hirai, S. Morita, S. Fukuhara, and M. Mishima. 2005. Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma. *Thorax* 60(4):277-81.

232. Wilson, J. W., X. Li, and M. C. Pain. 1993. The lack of distensibility of asthmatic airways. *Am Rev Respir Dis* 148(3):806-9.

233. Niimi, A., H. Matsumoto, R. Amitani, Y. Nakano, H. Sakai, M. Takemura, T. Ueda, K. Chin, H. Itoh, E. P. Ingenito, and M. Mishima. 2004. Effect of short-term treatment with inhaled corticosteroid on airway wall thickening in asthma. *Am J Med* 116(11):725-31.

234. Macklem, P. T. 1996. A theoretical analysis of the effect of airway smooth muscle load on airway narrowing. *Am J Respir Crit Care Med* 153(1):83-9.

235. Brusasco, V., and R. Pellegrino. 2003. Complexity of factors modulating airway narrowing in vivo: relevance to assessment of airway hyperresponsiveness. *J Appl Physiol* 95(3):1305-13.

236. de Jongste, J. C., H. Mons, I. L. Bonta, and K. F. Kerrebijn. 1987. In vitro responses of airways from an asthmatic patient. *Eur J Respir Dis* 71(1):23-9.

237. Bai, T. R. 1991. Abnormalities in airway smooth muscle in fatal asthma. A comparison between trachea and bronchus. *Am Rev Respir Dis* 143(2):441-3.

238. Black, J. L. 1991. Pharmacology of airway smooth muscle in chronic obstructive pulmonary disease and in asthma. *Am Rev Respir Dis* 143(5 Pt 1):1177-81.

239. Vincenc, K. S., J. L. Black, K. Yan, C. L. Armour, P. D. Donnelly, and A. J. Woolcock. 1983. Comparison of in vivo and in vitro responses to histamine in human airways. *Am Rev Respir Dis* 128(5):875-9.

240. Whicker, S. D., C. L. Armour, and J. L. Black. 1988. Responsiveness of bronchial smooth muscle from asthmatic patients to relaxant and contractile agonists. *Pulm Pharmacol* 1(1):25-31.

241. Goldie, R. G., D. Spina, P. J. Henry, K. M. Lulich, and J. W. Paterson. 1986. In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, beta-adrenoceptor agonists and theophylline. *Br J Clin Pharmacol* 22(6):669-76.

242. Fan, T., M. Yang, A. Halayko, S. S. Mohapatra, and N. L. Stephens. 1997. Airway responsiveness in two inbred strains of mouse disparate in IgE and IL-4 production. *Am J Respir Cell Mol Biol* 17(2):156-63.

243. Ammit, A. J., C. L. Armour, and J. L. Black. 2000. Smooth-muscle myosin light-chain kinase content is increased in human sensitized airways. *Am J Respir Crit Care Med* 161(1):257-63.

244. Jiang, H., K. Rao, A. J. Halayko, X. Liu, and N. L. Stephens. 1992. Ragweed sensitization-induced increase of myosin light chain kinase content in canine airway smooth muscle. *Am J Respir Cell Mol Biol* 7(6):567-73.

245. Skloot, G., S. Permutt, and A. Togias. 1995. Airway hyperresponsiveness in asthma: a problem of limited smooth muscle relaxation with inspiration. *J Clin Invest* 96(5):2393-403.

246. Coflesky, J. T., R. C. Jones, L. M. Reid, and J. N. Evans. 1987. Mechanical properties and structure of isolated pulmonary arteries remodeled by chronic hyperoxia. *Am Rev Respir Dis* 136(2):388-94.

247. Bramley, A. M., R. J. Thomson, C. R. Roberts, and R. R. Schellenberg. 1994. Hypothesis: excessive bronchoconstriction in asthma is due to decreased airway elastance. *Eur Respir J* 7(2):337-41.

248. Thomson, R. J., A. M. Bramley, and R. R. Schellenberg. 1996. Airway muscle stereology: implications for increased shortening in asthma. *Am J Respir Crit Care Med* 154(3 Pt 1):749-57.

249. Johnson, P. R. 2001. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 28(3):233-6.

250. Ward, C., D. P. Johns, R. Bish, M. Pais, D. W. Reid, C. Ingram, B. Feltis, and E. H. Walters. 2001. Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. *Am J Respir Crit Care Med* 164(9):1718-21.

251. Brackel, H. J., O. F. Pedersen, P. G. Mulder, S. E. Overbeek, K. F. Kerrebijn, and J. M. Bogaard. 2000. Central airways behave more stiffly during forced expiration in patients with asthma. *Am J Respir Crit Care Med* 162(3 Pt 1):896-904.

252. Pedersen, O. F., H. J. Brackel, J. M. Bogaard, and K. F. Kerrebijn. 1997. Wave-speed-determined flow limitation at peak flow in normal and asthmatic subjects. *J Appl Physiol* 83(5):1721-32.

253. Meiss, R. A. 1999. Influence of intercellular tissue connections on airway muscle mechanics. *J Appl Physiol* 86(1):5-15.

254. Bramley, A. M., C. R. Roberts, and R. R. Schellenberg. 1995. Collagenase increases shortening of human bronchial smooth muscle in vitro. *Am J Respir Crit Care Med* 152(5 Pt 1):1513-7.

255. Bai, A., D. H. Eidelman, J. C. Hogg, A. L. James, R. K. Lambert, M. S. Ludwig, J. Martin, D. M. McDonald, W. A. Mitzner, M. Okazawa, and et al. 1994. Proposed nomenclature for quantifying subdivisions of the bronchial wall. *J Appl Physiol* 77(2):1011-4.

256. Lambert, R. K. 1991. Role of bronchial basement membrane in airway collapse. *J Appl Physiol* 71(2):666-73.

257. Milanese, M., E. Crimi, A. Scordamaglia, A. Riccio, R. Pellegrino, G. W. Canonica, and V. Brusasco. 2001. On the functional consequences of bronchial basement membrane thickening. *J Appl Physiol* 91(3):1035-40.

258. Seow, C. Y., L. Wang, and P. D. Pare. 2000. Airway narrowing and internal structural constraints. *J Appl Physiol* 88(2):527-33.

259. Vanacker, N. J., E. Palmans, R. A. Pauwels, and J. C. Kips. 2002. Dose-related effect of inhaled fluticasone on allergen-induced airway changes in rats. *Eur Respir J* 20(4):873-9.

260. Saetta, M., P. Maestrelli, G. Turato, C. E. Mapp, G. Milani, F. Pivirotto, L. M. Fabbri, and A. Di Stefano. 1995. Airway wall remodeling after cessation of exposure to isocyanates in sensitized asthmatic subjects. *Am J Respir Crit Care Med* 151(2 Pt 1):489-94.

261. Saetta, M., A. Di Stefano, P. Maestrelli, N. De Marzo, G. F. Milani, F. Pivirotto, C. E. Mapp, and L. M. Fabbri. 1992. Airway mucosal inflammation in occupational asthma induced by toluene diisocyanate. *Am Rev Respir Dis* 145(1):160-8.

262. Sont, J. K., L. N. Willems, E. H. Bel, J. H. van Krieken, J. P. Vandenbroucke, and P. J. Sterk. 1999. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 159(4 Pt 1):1043-51.

263. Laursen, L. C., E. Taudorf, S. Borgeskov, T. Kobayasi, H. Jensen, and B. Weeke. 1988. Fiberoptic bronchoscopy and bronchial mucosal biopsies in asthmatics undergoing long-term high-dose budesonide aerosol treatment. *Allergy* 43(4):284-8.

264. Wilson, J. W. 1998. What causes airway remodelling in asthma? *Clin Exp Allergy* 28(5):534-6.

265. Laitinen, L. A., and A. Laitinen. 1996. Remodeling of asthmatic airways by glucocorticosteroids. *J Allergy Clin Immunol* 97(1 Pt 2):153-8.

266. Chetta, A., A. Zanini, A. Foresi, M. Del Donno, A. Castagnaro, R. D'Ippolito, S. Baraldo, R. Testi, M. Saetta, and D. Olivieri. 2003. Vascular Component of Airway

Remodeling in Asthma Is Reduced by High Dose of Fluticasone. *Am J Respir Crit Care Med* 167(5):751-757.

267. Paganin, F., V. Trussard, E. Seneterre, P. Chanez, J. Giron, P. Godard, J. P. Senac, F. B. Michel, and J. Bousquet. 1992. Chest radiography and high resolution computed tomography of the lungs in asthma. *Am Rev Respir Dis* 146(4):1084-7.

268. McParland, B. E., P. T. Macklem, and P. D. Pare. 2003. Airway wall remodeling: friend or foe? *J Appl Physiol* 95(1):426-34.

269. Ayres, J. G., J. F. Miles, and P. J. Barnes. 1998. Brittle asthma. *Thorax* 53(4):315-321.

270. Yamasaki, A., K. Tomita, H. Sano, M. Watanabe, H. Makino, J. Kurai, Y. Hitsuda, and E. Shimizu. 2003. Measuring subepithelial thickness using endobronchial ultrasonography in a patient with asthma: a case report. *Lung* 181(3):115-20.