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Airway remodelling in asthma

Aspects of airway wall thickness and extracellular matrix production

by

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ABSTRACT

Asthma is a chronic inflammatory disorder of the airways which leads to episodic bronchospasm. A proportion of sufferers have non-reversible structural changes within their airway wall which results in a permanent airflow defect and more severe symptoms. Remodelling is thought to result in overall thickening of the airway wall. This work looks at remodelling by measurement of airway dimensions and assessing the role of potential contributors to the process, namely collagens, proteoglycans and galectins.

Subjects were recruited and characterised through a clinical history, skin prick testing, exhaled nitric oxide and a PC_{20} to methacholine. They underwent bronchoscopy and subgroups had measurement of airway wall thickness via endobronchial ultrasonography or computed tomography. Bronchoalveolar lavage fluid, epithelial cell brushings and bronchial biopsies were also obtained at bronchoscopy. A subgroup of mild asthmatics underwent allergen challenge with retrieval of BALF pre -and post-challenge. Immunohistochemistry for collagens 1 and 3 and galectin-3 was performed on the endobronchial biopsies. Fibroblasts were grown from endobronchial biopsies and stimulated with $TGF\beta_2$ and TNFα. Proteoglycan message for biglycan, decorin, lumican and versican was measured via PCR and protein output for decorin and versican by the same cultured fibroblasts via ELISA. Galectin-3 mRNA was measured in bronchial brushings and levels in lavage fluid were also analysed pre-and post-allergen challenge.

Collagen 1 and 3 showed increased immunostaining in asthma, which correlated with computed tomographic measures of airway wall thickness within the whole group but not within the asthma group on its own.

Endobronchial ultrasound image measurements of inner wall area (consisting of epithelium and submucosa) were found to be significantly thicker in more severe asthmatics especially when considering the subset of asthmatics with a non-fully reversible airflow defect. These measurements also indicate that the same subgroup of asthmatics have wider lumina.

Unstimulated primary central airway fibroblasts cultured from subjecs with or without fixed airflow obstruction did not show any significant variation in proteoglycan mRNA output. Stimulation with TNFα did not appear to have any appreciable impact on proteoglycan output. Versican mRNA and protein and biglycan mRNA were upregulated by $TGF\beta_2$whilst decorin mRNA and protein were downregulated.

The relative production of galectin-3 by the epithelia of non-asthmatics, mild and severe asthmatics was examined. No significant differences were evident in either the mRNA in bronchial brushings or protein levels as determined by immunostaining of biopsies as well as bronchoalveolar lavage levels. There was a significant increase in BALF galectin-3 after allergen challenge.

Fixed airway obstruction is associated with increased thickness of the submucosal layer of the airway wall as well as the airway lumen. There is no association between fixed airway obstruction and proteoglycan output by central airway fibroblasts. Epithelial galectin-3 is not upregulated in stable asthma but is increased in BALF post-allergen challenge.

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DECLARATION OF AUTHORSHIP

I, David Sammut, declare that the thesis entitled "Airway remodelling in asthma – Aspects of airway wall thickness and extracellular matrix production" and the work presented in the thesis are both my own, and have been generated by me as a result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission.

Signed: .....

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LIST OF ABBREVIATIONS

%pred	percent of predicted
A2	phospholipase A2
AAIR	Asthma, Allergy and Inflammation Research charity
ACQ	asthma control questionnaire
AEC	3-amino-9-ethylcarbazole
A_{ext}	external area
A_{inner}	inner area
A_{int}	internal area
αSMA	alpha smooth muscle actin
ART	Aerosol Resistant Tips
A_{sec}	sector area
ASM	airway smooth muscle
ATS	American Thoracic Society
BAL or BALF	bronchoalveolar lavage fluid
BD	bronchodilator
BDP	beclomethasone dipropionate
BSA	bovine serum albumin
BTS	British Thoracic Society
CD	cluster of differentiation nomenclature of leukocyte cell surface antigens
cDNA	complementary deoxyribonucleic acid

C/EBPα	CCAAT/enhancer binding protein α
COPD	chronic obstructive pulmonary disease
CRD	carbohydrate-recognition domain
Ct	threshold cycle in a polymerase chain reaction
CT	computed tomography
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EBUS	endobronchial ultrasound
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERS	European Respiratory Society
F	female
FACITs	fibril-associated collagens with interrupted triple helices
FBS	foetal bovine serum
FeNO	fraction of exhaled nitric oxide
FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
GAG	glycosaminoglycan

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GINA	Global Initiative for Asthma
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMA	glycol methacrylate
GP	general practitioner
GPC	Golgi-to-plasma membrane carrier
H_2O_2	hydrogen peroxide
HBSS	Hank's balanced saline solution
HRCT	high resolution computed tomography
HSP	heat shock protein
Hz	Hertz
I	intermittent asthma
ICAM-1	intercellular adhesion molecule-1
ICS	inhaled corticosteroid
IgE	immunoglobulin E
IL	interleukin
IU	international units
IWA	inner wall area
LABA	long-acting β_2 agonist
LAP	latency associated protein
LCC	large latent complex
LED	light-emitting diode
LREC	local research ethics committee
LRR	leucine-rich region

LTBP	latent TGFβ-binding protein
M	male
MACITs	membrane-associated collagens with interrupted triple helices
MDI	metered dose inhaler
MEM	minimum essential medium
MMLV	Moloney murine leukaemia virus
MMP	matrix metalloproteinase
MoP	moderate persistent asthma
MP	mild persistent asthma
NEAA	non-essential amino acids
NF-κB	nuclear factor- kappaB
NO	nitric oxide
OCS	oral corticosteroid
oligo-dT	oligodeoxythymidylic acid
p	probability value
PBS	phosphate buffered saline
PC_{20}	methacholine concentration causing a 20% drop in FEV_1
PCR	polymerase chain reaction
PEF	peak expiratory flow
PEFR	peak expiratory flow rate
PIWA	percentage inner wall area
PWA	percentage wall area
qPCR	quantitative polymerase chain reaction

RANTES	regulated upon activation normal T cell expressed and secreted
rDNase I	recombinant deoxyribonuclease I
RBM	reticular basement membrane
RNA	ribonucleic acid
Rnase	ribonuclease
RPM	revolutions per minute
RT	reverse transcription
RV	residual volume
SLRP	small leucine-rich proteoglycan
SP	severe persistent asthma
StABC-HRP	streptavidin-biotin complex horse radish peroxidase labelled complex
T/D	thickness-to-diameter ratio
TACE	TNFα converting enzyme
Taq	Thermus aquaticus
TBS	tris-buffered saline
TGFβ	transforming growth factor beta
$TGF\beta_2$	transforming growth factor beta isoform 2
T_H1	T helper cell type 1
T_H2	T helper cell type 2
TIFF	tagged image file format
TIMP-1	tissue inhibitor of metalloproteinase-1
TMB	tetramethylbenzidine

TNFα	tumour necrosis factor alpha
UBC	ubiquitin C
UK	United Kingdom
US	United States
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

CHAPTER 1

Introduction

1.1 Asthma definition

Asthma is defined in the 2009 global initiative for asthma (GINA) report [1] as "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with 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 within the lung that is often reversible either spontaneously or with treatment."

A complex, and still incompletely understood, interplay of cells and mediators in the bronchial wall is thought to lead to increased airway hyperresponsiveness resulting in airflow limitation via a number of mechanisms. In the short term this causes acute bronchoconstriction, bronchial wall oedema and stimulation of mucus-secreting cells resulting in mucus plugging. In the long term, incompletely reversible structural wall changes called airway wall remodelling set in.

A collection of symptoms characterise asthma, including shortness of breath, wheezing, chest tightness and cough. These symptoms are typically episodic reflecting the transient changes in the calibre of the airways. However with airway remodelling, there are structural changes in the wall which preclude complete reversibility of the airflow limitation and consequently generate persistent symptomatology. It is often difficult to predict which patients will go on to develop more severe airway changes with fixed airflow limitation and there are some suggestions that there may be radical differences in pathophysiology between asthma with and without marked airway remodelling [1].

1.2 Asthma epidemiology

Asthma is a very common disease, particularly in industrialised countries. In fact its prevalence is increasing worldwide – there are a calculated 300 million people in the world suffering from asthma [1]. According to US epidemiological surveillance data, for the period 2001–2003, there were an estimated 20 million asthmatics in the US and around 4000 deaths per year were attributable to asthma [2]. The "BTS Burden of Lung Disease 2006" reports that asthma accounted for a total of 1 381 deaths (502 males, 879 females) in 2004 in the UK and 70 907 hospital admissions in England [3].

With increased awareness and more standardised management, the number of hospitalisations for asthma is in fact on the decline, reflecting a better control of the disease [2]. However a subgroup of patients with more severe disease (between 5 and 10% of asthmatics) appears to account for a disproportionate fraction of admissions, morbidity and costs [4]. Although no studies have specifically looked at this, it is not unreasonable to suppose that this group is, at least partly, made up of those subjects who have pronounced airway remodelling and on whom current asthma medications seem to have little impact.

1.3 Predisposition to asthma

As in most other diseases, asthma is thought to arise through the action of environmental factors on a genetically susceptible host.

There is no single genetic abnormality directly associated with asthma, although clinical experience has long ago underlined the relevance of a positive family history. A number of susceptibility genes have been identified to date with diverse functions being shown or hypothesised for them. Genetic associations are usually identified either by genome-wide association studies, positional cloning or by

investigation of candidate genes selected on the basis of likely biological relevance. These include genes involved in pathways of atopy and inflammation (*IL1RL1*, *IL-4*, *IL4RA, IL13*, *FCER1B*, *TNFA*, *TLE4*, *IL-33*), lung remodelling and angiogenesis (*ADAM33*), smooth muscle contractility (*PDE4D*), antigen presentation (*HLA-DRB1*, *HLADQB1*), and pharmacotherapeutic response (*ADRB2*). For others the function remains elusive (*ORMDL3*, *DPP10*) [5, 6]. Lung development, bronchial hyperresponsiveness, propensity to atopy, ability to generate inflammatory mediators can all be affected by the implicated genes. Furthermore they can act, possibly synergistically, at different points in the natural history of asthma [7].

Atopy, the production of abnormal amounts of immunoglobulin E to common environmental allergens, is strongly associated with asthma. The mechanism underlying atopy involves sensitisation of mast cells to specific allergens and subsequent repeated activation on re-exposure. The release of various mediators by the activated mast cell results in a cascade of events which fuel the inflammatory response. Studies report figures between 30 and 40% of asthma as being attributable to atopy [8, 9].

Environmental triggers including tobacco smoking, occupational sensitizers, ambient pollution and possibly diet [1] can contribute to the development or preservation of an asthmatic response. Features of the habitat in utero and in the peri-natal period, such as maternal smoking [10] and diet during pregnancy [8-10] as well as intra-uterine growth and birth weight [11] have been shown to determine the later development of asthma.

There is controversy about the impact of early life exposure to infection. On one hand, it is postulated to skew the child's immune response away from the allergic phenotype. However there is also evidence that certain childhood infections, particularly respiratory syncytial virus, predispose to asthma [12, 13] by

sensitising the epithelium to future infections [14]. These findings may not be mutually exclusive as the site of infection and the age at which it occurs could have different outcomes. In a cohort of Australian children, up to 3 upper respiratory infections in the first year of life conferred protection against risk of asthma at age 6, whilst the development of lower respiratory tract infections associated with wheeze in infancy increased the risk [15].

1.4 Asthma pathophysiology

Despite the fact that information about asthma pathophysiology is abundant, an all-encompassing outline of its aetiology remains elusive. One of the reasons for this is the fact that asthma is a very heterogeneous disease with numerous clinical subtypes which are often difficult to classify reproducibly, and in which different pathophysiological processes may be relevant. A lot of the gross anatomical and histopathological changes seen in asthmatic lungs have been established for more than half a century, from post-mortem studies of patients dying in status asthmaticus. The molecular biology of the disease is, on the other hand, still a work in progress.

1.4.1 Sputum

Simple microscopy of sputum from asthmatics mirrors the changes occurring in the airways where it is produced. The sputum is, typically, very rich in eosinophils, and contains Charcot-Leyden crystals (galectin-10, a product of eosinophils) [16], spiral twists of condensed mucus (Curschmann spirals) and clumps of shed epithelial cells (Creola bodies).

1.4.2 Macroscopic pathology

Gross pathology of the lungs of subjects who have died following an asthma exacerbation shows hyperinflated lungs, airway plugging with thick tenacious sputum, small foci of collapse and areas of subpleural fibrosis especially in the periphery of the upper lobes [17–20]. These features are also manifest, to a lesser degree, in the lungs of asthma sufferers where asthma was not the direct cause of death.

1.4.3 Microscopic pathology

Histopathology of the airways is characterised by increased mucus in the lumen, inflammatory oedema and muscular hypertrophy of the bronchial wall [21]. Changes are most marked in the bronchi and proximal bronchioles.

There is detachment of superficial epithelial cells from basal cells, and this desquamated epithelium together with an exudate of eosinophils and mucus contributes to airway plugging [22].

Goblet cell hyperplasia is noted in the epithelium as well as enlarged bronchial glands. Increased mucus production is thought to result from a combination of this increased capacity for output as well as stimulation by exposed intraepithelial nerves [23].

There is an increased presence of inflammatory cells in the asthmatic bronchial wall which participate in the inflammatory cascade occurring in the airways. These include eosinophils [24], lymphocytes, in particular T helper cells [25], mast cells [26, 27] and neutrophils. The role of the latter, although less evident, is being reappraised in view of the fact that they seem to be more abundant in fatal cases of asthma [28, 29].

Another frequently encountered, although by no means specific, feature is basement membrane thickening [30]. The increased thickness is mostly in the lamina reticularis and is associated with aggregation and proliferation of myofibroblasts [31] resulting in increased collagen and tenascin deposition [32] in the area.

The airway smooth muscle (ASM) bulk increases as a consequence of a combination of hyperplasia and hypertrophy [33]. Together with the intramural oedema, this contributes to airway wall thickening.

1.4.4 Molecular biology

The pathophysiological pathways of asthma are far from unequivocally established. Combinations of genetic and environmental factors initiate, potentiate and modify the airway changes.

Figure 1.1 outlines a, necessarily simplified, overview of the sequence of events which are thought to lead to the establishment and self-maintenance of inflammation in asthmatic airways.

Bronchial epithelium is a site of continuous allergen exposure and for this reason a rich network of antigen presenting dendritic cells is found throughout the conducting airways as well as within the interstitium and lymph nodes. Dendritic cells are closely associated with the bronchial epithelium both through cytokine cross-talk as well as the presence of dendritic extensions to the luminal border in between epithelial cells. One of their functions is to identify inhaled allergen (as well as any microbial, physical or toxic injury to the airway), ingest it and migrate to local lymph nodes where they present it to T and B cells. There is, however, increasing appreciation of the complexity of dendritic cell populations within the lung and their involvement not just in promoting T_H2 cell polarisation but also the induction of tolerance through regulatory T cells [34, 35].

Antigen presentation occurs within the lymph nodes, where T cells are activated via major histocompatibility complex class II molecules and co-stimulatory signals. These T cells in turn activate B cells via interleukins (IL) - 13 and 4, to start to produce immunoglobulin E [34]. Under the influence of these cytokines, B cells isotype switch from IgM and IgG to IgE and IgG4 [36, 37].

Soluble IgE enters the circulation and binds via low-affinity IgE receptors to a number of cells including lymphocytes, eosinophils and macrophages, as well as platelets [38]. More importantly, it undergoes high-affinity binding to mast cells which are found in local tissue, where they constitute up to 20% of inflammatory cells. On binding allergen, mast cells become activated through cross-linking of their high-affinity IgE receptors. They secrete a number of pre-formed mediators including histamine, proteases and chemotactic factors. Denovo synthesis of other inflammatory mediators such as leukotrienes and prostaglandins is also boosted [39]. These are responsible both for the immediate bronchospasm and oedema, as well as the more long-term recruitment of other inflammatory cells.

On the basis of their abundance in bronchoalveolar lavage fluid, bronchial biopsies and peripheral blood [40], one of the key cells in asthma is the eosinophil. IL-5, produced by both mast cells and T cells, promotes the release of mature eosinophils from the bone marrow. Once in the circulation, they migrate and localise to the lung tissue via interactions between eosinophil cell surface molecules such as integrins and endothelial cell adhesion molecules expressed in lung blood vessels. Within the lung, the activated eosinophil releases a number of mediators which are contributory to prolonged airway inflammation and injury. Despite the fact that eosinophil levels in various tissues in asthmatic subjects have been found to correlate with clinical severity and bronchial hyperresponsiveness, attempts at therapeutically targeting IL-5, although successful in reducing eosinophil counts, have failed to translate into clinical

efficacy [41]. This suggests the possibility that a number of parallel processes are at play in asthma some of which are eosinophil-independent [42].

Activated lymphocytes are also present in bronchial biopsies of asthmatic subjects. These appear to be predominantly CD4+ helper T (T_H) cells. A number of subsets of T_H cells are described on the basis of their cytokine profile. The role of type 1 (T_H1) and type 2 (T_H2) helper T cells has been the focus of a lot of work in asthma. T_H1 cells are responsible for cellular defence mechanisms mainly through the generation of IL-2 and interferon γ, whilst T_H2 cells produce predominantly interleukins-4, 5, 6, 9 and 13 and mediate allergic inflammation. The two groups reciprocally inhibit each other so that, for instance, interferon γ inhibits the differentiation of T_H2 cells.

When a non-asthmatic individual is exposed to an allergen, a T_H1 response is typically produced [43]. On the other hand, the bronchial mucosa of asthmatics appears to have an excess of T_H2 cells [44, 45], and the resulting imbalance of T_H1 and T_H2 cells appears to have a pivotal role in asthma. By virtue of the cytokines involved, preferential T_H2 activation results in a pro-allergic milieu with increased IgE production, mast cell and eosinophilic activation [46].

The immune system of the newborn is initially skewed towards a T_H2 phenotype [47], but repeated exposure to infection, which promotes T_H1 differentiation, rectifies this discrepancy. The “hygiene hypothesis” [48] attributes the increased incidence of asthma in industrialised countries to the reduced exposure of infants to environmental T_H1 primers such as bacterial and viral antigens in early life [49, 50]. Not all data fit nicely within the confines of this theory – so, for example, there has been an increase in T_H1-autoimmune diseases in industrialised countries as well as atopy and the T_H2-skewed helminthic infections appear to be protective against the development of allergy [51].

A proportion of subjects have so called non-atopic asthma (anything between 20 and 80% is quoted for different populations [52-54]), where there is no demonstrable sensitivity to common aeroallergens and less likelihood of a positive family history of atopy. Despite this, their asthma is still thought to be driven by inappropriate IgE production, a similar inflammatory cell infiltrate and an elevated expression of the same key cytokines [55-57].

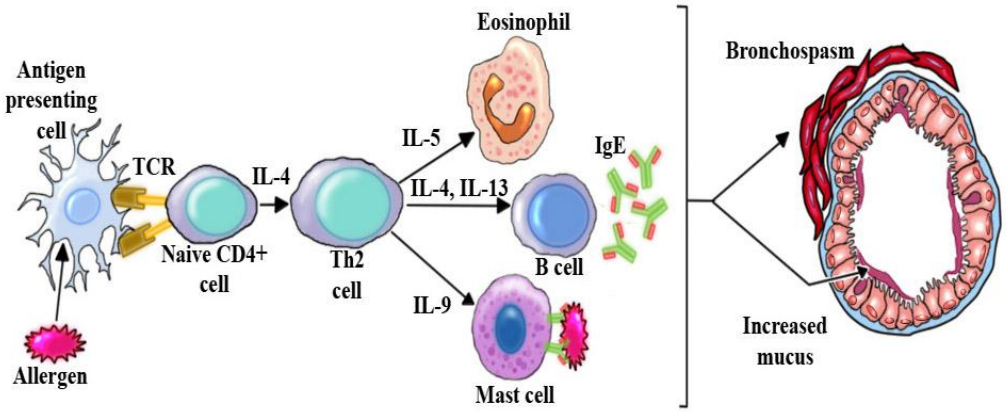

Figure 1.1. Main cells and cytokines involved in asthma pathophysiology. Adapted from [58].

1.5 Airway remodelling

Airway remodelling refers to structural changes that are manifest in essentially all asthmatic airways but are particularly marked in a group of asthmatics who have a permanent airflow limitation defect and often have persistent rather than episodic symptoms [59].

The presence of airway wall changes in asthma has been described for many years. Sir William Osler in his milestone work 'The Principles and Practice of Medicine' published in 1892 talks about "hyperaemia and turgescence of the mucosa of the smaller bronchial tubes" as a hallmark of asthma [60].

The structural changes of remodelling include epithelial goblet cell hyperplasia, subepithelial fibrosis, smooth muscle cell hyperplasia and hypertrophy and proliferation of blood vessels and nerves [30, 61].

Although the concept of remodelling is not unchallenged, it is an area of increasing research. The intuitive conclusion that remodelling occurs as a consequence of more prolonged and more severe inflammation is gradually giving way to the idea that it is more of a parallel process that occurs to varying degrees in different asthmatics.

1.5.1 Epithelial remodelling

The epithelium is the first point of contact between the external environment and bronchial tissue. In asthma, a genetically susceptible epithelium is postulated to be more injury-prone and has inefficient and abnormal healing responses [62]. The chronic wound milieu that is consequently created is thought to drive the structural changes both in the epithelium itself as well as in the underlying submucosa [63].

Epithelial repair involves a well-co-ordinated sequence of events aimed at efficiently closing a breach in the body's defences. In vitro studies of epithelial repair models show that the initial step involves a change in the nearby epithelial cells which undergo an epithelial-to-mesenchymal transition. This involves a downregulation of tight junction formation and upregulation of extracellular matrix (ECM) protein and metalloprotease expression. This facilitates migration of cells and increases their synthetic capabilities to form a provisional barrier [64].

Under the influence of cytokines released by the epithelial cells, fibroblasts migrate and proliferate to form a subepithelial sheath and synthesise a provisional matrix [65]. In the meantime, epithelial cells proliferate with increased goblet cell differentiation and ciliogenesis – the increased mucus production and cilia providing a further check to invasion [66]. Once epithelial repair has been finalised, these changes are reversed.

In asthma there is increased epithelial injury as shown by direct evidence of epithelial desquamation from bronchial biopsies [67] and post-mortems [68] as well as indirect evidence of injury and repair (release of HSP-70 [69], increase in EGFRs [70, 71] and CD-40 [72]). Asthmatic epithelium has been shown to be more vulnerable to injury including oxidant-induced apoptosis [62] and viral infection [73]. Tight junctions are an important component of the barrier function of the epithelium and bronchial epithelium in asthmatics appears to have an impaired ability to form effective tight junctions [74].

Apart from a higher vulnerability, the epithelium has an impaired repair response – there is a reduced output of p21 (waf) cyclin dependent kinase inhibitor, a cell cycle inhibitor, which could affect the ability of the epithelium to replace shed cells [75]. The repairing epithelium shows an abnormal output of various cytokines including PGE_2, IL-6 [76], and $TGF\beta_2$ [70] which in turn can affect various cells of the bronchial wall.

Increased quantities of mucus are found in the airways of asthmatics, particularly the more severe phenotypes and cases of fatal asthma. Hyperplasia of goblet cells [77] and an increase in size of the submucosal glands [78, 79] are thought to account for this mucus hypersecretion. This not only results in an increased output of mucin, but even the nature of the mucin is different with an increased presence of the large, cysteine-rich, gel-forming MUC5AC and the low-charge glycoform of MUC5B gene products, being produced [80-82]. Although there are

documented changes in mucin type and glycosylation, there is no evidence to suggest any functional differences between these different gels [83]. A number of inflammatory mediators, together with neural mechanisms, have been shown to have potential for affecting goblet cell hyperplasia, altering mucin gene expression or facilitating mucin secretion [84].

Central to the idea of airway remodelling is the concept that there are interactions between the epithelium and the sub-epithelial cells. The epithelial-mesenchymal trophic unit [85], as this is commonly referred to, is thought to represent, under some aspects, a re-activation of the processes occurring during embryonic lung development. This leads to the release of pro-inflammatory mediators by the epithelium that brings about inflammatory cell infiltration but also contributes a number of pro-fibrogenic growth factors which act on the submucosal cells [86, 87].

1.5.2 Reticular basement membrane thickening

The subepithelial basement membrane consists of two main components. The basal lamina or true basement membrane, on which the basal epithelial cells rest is around 80nm thick and only distinguishable by electron microscopy. Beneath it lies the lamina reticularis, or reticular basement membrane (RBM), which is about 4 μm in thickness. The basal lamina consists primarily of collagen 4 produced by the overlying epithelial cells, whilst the RBM is made by the structural cells of the subepithelium from collagens 1, 3 and 5.

Increased thickness of the reticular basement membrane in asthma has been documented in a number of studies [30, 65, 88, 89]. It is a result of increased deposition of interstitial collagens [30, 88] tenascin [90] and laminins [91, 92].

RBM thickening has been documented as early as 4 years of age and seems to be maximally thickened by late childhood to mid-teens [93-95]. The association with

disease characteristics and natural history is less clear. No evidence of association with asthma duration [96, 97] has been demonstrated and although some studies have shown a relationship to asthma severity [97], others have failed to do so [94]. A correlation with FEV_1 and airway hyperresponsiveness [89, 97] has been shown by some groups but not by others [98]. More recently, atopy has emerged as an important determinant of reticular basement membrane thickening. A number of studies have shown that the presence of atopy in asthma is correlated with a thicker RBM [99], and indeed RBM changes are found in the presence of atopy or rhinitis without asthma [100-102].

1.5.3 Airway smooth muscle hyperplasia and hypertrophy

An increase in airway smooth muscle mass is one of the more obvious histopathological changes in the bronchial wall of asthmatics. This has implications not only by way of the resulting increased contractile potential, but also in light of the increasing evidence of the potential for airway smooth muscle cells to secrete chemokines and cytokines. Airway smooth muscle cells are being increasingly shown to have secretory and immunomodulatory functions in vitro, shifting their perceived role in asthma from one of end effector cell to a more central one [103].

An increase in airway smooth muscle mass has been demonstrated in post-mortem studies [78, 96, 104-106] and endobronchial biopsy studies [107, 108]. The difficulty associated with obtaining endobronchial biopsies of adequate thickness to permit studying the ASM means there is less data from this source. Whether the increased mass is due to hypertrophy or hyperplasia of the smooth muscle cells is still unclear. Endobronchial biopsies from severe asthmatics, revealed an increased ASM cell diameter compared to controls in one study [107] whilst another study involving mild-to-moderate asthmatics showed an increase in number without a size change [108]. These different findings are possibly

determined by the asthma subset investigated. In fact, Ebina *et al* [109], in post-mortem studies on 10 asthmatics, identified two patterns of ASM increase. In one group of asthmatics, which they denote as type I, there was an increase in ASM mass only in the central bronchioles resulting from cell hyperplasia. In the other group, the type II pattern, there was an increase in ASM throughout the bronchial tree with evidence of hypertrophy of the cells.

The observed increase in ASM cell numbers could be a consequence of increased proliferation [110], reduced apoptosis [111] or migration of cells (possibly from the myofibroblast pool) [103]. Mitogens and growth factors are released by the epithelium or in response to anti-apoptotic signals from the surrounding extracellular matrix. Apart from this, there is some evidence of intrinsic differences in the ASM cells of asthmatics. Roth *et al* have shown that asthmatic ASM cells lack the anti-proliferative transcription factor C/EBPα (CCAAT/enhancer binding protein α) [112].

There is no difference in contractile power between human ASM cells from asthmatic or non-asthmatic airways [113, 114], although there is evidence of an increased maximal shortening capacity and velocity in asthma [115]. Presumably increased muscle mass is both able to cumulatively generate more force and at the same time cause relatively more luminal narrowing due to the increased thickness of the airway wall, resulting in bronchial hyperresponsiveness. Other relevant abnormalities attributed to ASM include impairment in relaxation in response to β_2-agonists [116] and a reduced capacity for relengthening after being subjected to force fluctuations such as occurs on deep inspiration [117].

ASM cells in culture have been shown to be able to synthesise a number of pro-inflammatory chemokines and cytokines. These include eotaxin (eosinophil chemoattractant) [118], RANTES (regulated upon activation normal T cell expressed and secreted – an eosinophil, memory T cell and monocyte

chemoattractant) [119], IL-1β (pro-inflammatory and pro-fibrotic cytokine), IL-5 (promotes eosinophil survival) [120], IL-11 (pro-fibrogenic) and IL-6 (promotes B- and T-cell maturation and activation) [121], granulocyte-macrophage colony-stimulating factor (GM-CSF – enhances eosinophil and neutrophils function and survival) [122]. These are of relevance in driving the pro-inflammatory milieu, acting both in an autocrine and a paracrine fashion.

They are also able to elaborate a number of growth factors, such as TGFβ [123], and VEGF (vascular endothelial growth factor) [124], which have roles in immunomodulation, fibrosis and angiogenesis. Adhesion molecules, VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1), which can facilitate binding of various cells including T cells and eosinophils, have been shown to be expressed on ASM cells [125].

There is no extensive evidence showing a difference between the synthetic capabilities of asthmatic and non-asthmatic ASM cells. In culture, human ASM cells, irrespective of their source, undergo morphological changes to take on a more synthetic role with an upregulation of cell organelles. Although there is no evidence that this occurs in vivo, in view of the documented increased presence of growth factors and mitogens in the asthmatic airway it is not hard to imagine that these cells may be pushed towards a pro-synthetic phenotype [126].

1.5.4 Extracellular matrix changes

Increases in both the collagen [30, 65, 96, 127-129] and proteoglycan components [130-132] of the extracellular matrix have been shown in asthma. These changes determine many of the physical properties of the airway as well as influencing structural and inflammatory cell migration, proliferation and function. Further details in Section 1.8.

1.5.5 Remodelling in the adventitia

The adventitia makes up the connective tissue external to the smooth muscle bundles. There is evidence that this part of the bronchial wall also increases in thickness in asthma [78, 133]. This may diminish the transmission of static and dynamic forces from the lung parenchyma to the airway, meaning less dampening of airway smooth muscle contraction.

1.5.6 Timeline of remodelling

The initial models of remodelling were based on the hypothesis that the chronic inflammation in the bronchial wall, punctuated by acute exacerbations, leads to structural changes as a result of disordered repair. However, more recent work, particularly in children, has shown that remodelling within the airway is probably occurring in parallel with inflammation, and may even possibly antecede it [134].

Evaluation of bronchial biopsies in children with difficult asthma has shown that they have reticular basement membrane thickening equivalent to that found in adults with asthma [93]. Other investigators have demonstrated that in asthmatic children, the epithelium is already damaged [94] and expresses markers of injury without eosinophilic inflammation suggesting that airway remodelling is occurring independently of inflammation [135]. Pohunek *et al* followed up children with early respiratory symptoms not formally diagnosed as asthma, and found that there was increased lamina reticularis thickness in the subset of children who later developed asthma [95].

In a group of adult subjects, subepithelial layer thickness was associated with the more symptomatic subjects but had no relation to asthma duration [97].

1.5.7 Central and peripheral remodelling

Most data about airway remodelling relate to the central airways which are, obviously, more easily sampled. However there is evidence to suggest that the changes seen in the more proximal parts of the bronchial tree are also true of the more distal segments. Increased airway resistance has been measured in the peripheral airways of asthmatics [136] particularly in those having airflow obstruction [137], as has significant bronchial hyperresponsiveness [138]. Investigators have described increased inflammatory cell infiltration in distal airways [139], which may actually be more pronounced than that encountered more centrally [54, 55]. There is also evidence that in fatal asthma there is increased smooth muscle area and subepithelial collagen in the small airways of asthmatics compared to non-asthmatic controls [96].

1.5.8 Does remodelling equate with severity?

Studies have looked at different components of airway remodelling and tried to correlate them with asthma severity based on clinical classifications or physiological parameters.

Percentage goblet cell area is increased in autopsies of cases of fatal asthma compared to non-fatal asthma [79] and the mucous glands are larger in severe persistent asthma [107]. Reticular basement thickness has shown positive correlation with asthma severity [97], and negative correlation with spirometric measures of airways obstruction [97, 101, 140] .The relationship with airways hyperresponsiveness is more equivocal with some studies describing a positive [97, 140] and others a negative relationship [101]. Subepithelial collagen has been found to correlate with asthma severity (categorised by FEV_1) [141] and with airway responsiveness [142]. Increased airway smooth muscle area has been

described in post-mortems of fatal asthma [78] as well as in bronchial biopsies from severe persistent asthma [107] compared to milder forms of asthma.

1.5.9 Airway remodelling – pros and cons

Airway remodelling can be seen as a self-protective physiological response which, when taken beyond a certain limit, can translate into debilitating disease.

On the plus side, stiffening of the airways can help minimise excessive airway hyperresponsiveness [143] by constraining contraction of the smooth muscle cells as well as providing an elastic load. Increased vascularity, which is part of the remodelling process, may presumably increase the elimination of inflammatory mediators and contractile agonists. Goblet cell hyperplasia, through increased mucus production, can also provide a barrier to offending toxins or allergens.

The remodelling can, however, be detrimental in a number of ways. The thickened submucosa and epithelium amplify any narrowing effect of airway smooth muscle shortening. The increased smooth muscle mass may also have increased ability to generate radial stress. The smooth muscle layer is usually coupled to the surrounding lung parenchyma via the adventitial layer which transmits elastic recoil during changes in lung volume. With increased thickness of the adventitial layer, the transmission of elastic recoil forces to the airway may become inefficient allowing more narrowing. Airway smooth muscle is also thought to adapt to a shorter length by forming 'long-term' latch bridging between actin and myosin, while still retaining the ability to generate the same degree of force [144].

1.5.10 Is airway remodelling reversible?

The data available on the reversibility of remodelling are minimal, and in most cases confined to in vitro experimentation and animal models. Most of the studies, nevertheless, do show that many of the components of airway

remodelling are indeed reversible with available therapy, particularly corticosteroids. There are relatively few studies extrapolating this to humans.

Restoration of epithelial structure was shown in a 10-year follow-up study in a small number of severe asthmatics with the use of inhaled steroids [145], whilst an increase in number of ciliated cells was observed in a group of newly diagnosed asthmatics treated with inhaled budesonide over 3 months [146]. Another study reported decreased numbers of goblet cells in the epithelium of asthmatics who received concomitant allergen and steroid when compared to those receiving placebo [147].

Reticular basement membrane thickening has received more attention due to its perception as a hallmark of remodelling. However results are a bit more difficult to interpret, partly due to the different methodologies used. Reduction in RBM thickness with steroid treatment over between 6 weeks to one year was demonstrated in some studies [148-152]. A couple of other studies [128, 153] where the duration of treatment was shorter, 4 and 8 weeks respectively, failed to show this effect.

There are no longitudinal human studies on the effects of treatment on airway smooth muscle mass. Intranasal fluticasone during allergen challenge inhibited thickening of the smooth muscle layer in a murine model of chronic asthma [154]. Another group failed to show any effect of intraperitoneal dexamethasone on preventing peribronchial smooth muscle thickening in mice when co-administered with allergen [155], although when given in established remodelling, together with allergen avoidance, the ASM changes were reversed, in another study [156].

Other effects of corticosteroids include reducing bronchial wall vascularity [157] and myofibroblast numbers [155].

Information on other treatment modalities apart from corticosteroids is even scantier. Leukotriene receptor antagonists, β_2-agonists, tiotropium and theophylline have all been shown to potentially impact on airway remodelling [158]. The most direct modality of reversing ASM changes is bronchial thermoplasty involving ablation of the smooth muscle layer, which had shown a lot of promise in early trials, but has been less impressive in subsequent work [159].

1.6 Fibroblasts and myofibroblasts

Fibroblasts are connective tissue cells that have the ability to secrete collagen, fibronectin and tenascin as well as other components of the extracellular matrix. Their numbers are increased in asthma [65, 89] and they can be stimulated to secrete extracellular matrix proteins by a number of mediators especially Transforming Growth Factor β (TGFβ). TGFβ_2 release is increased in epithelial cell wound models [70].

Myofibroblasts, first described by Gabbiani in 1971 [160] represent differentiated fibroblasts, having similar secretory functions with added contractile ability. They are more abundant in asthma, specifically in the lamina reticularis of the thickened basement membrane [65, 88].

The myofibroblast (Figure 1.2) is thought to have a central role in physiological tissue repair processes and consequently also in the pathological persistence of the repair phenotype which results in remodelling. It has both contractile and synthetic functions enabling wound approximation as well as inflammatory cytokine and extracellular matrix (ECM) protein production.

The presence of myofibroblasts in tissue is usually associated with some form of tissue repair. Although their presence has been documented in non-repairing

tissue including bone marrow and alveolar septa, it is not understood what role, if any, they are covering in these situations. They have also been described in embryological tissue [161].

The study of myofibroblast molecular biology is not helped by the difficulty in identifying satisfactory markers that distinguish it from fibroblasts and smooth muscle cells. The incorporation of alpha-smooth muscle actin (αSMA) in the cell stress fibres is usually taken to define the transition of a fibroblast to a myofibroblast [162], however this does not distinguish it from a smooth muscle cell. Other cell features that change in the transition to a myofibroblast include modifications in the adherens junctions. The stress fibres of adjacent myofibroblasts are connected at adherens junctions via trans-membrane cadherins. Whilst N-cadherin appears to be the most common cadherin in fibroblasts, OB-cadherins increase with differentiation into myofibroblasts [163].

In the presence of injury, myofibroblasts are thought to arise from a number of possible sources. The most studied is the transformation of local resident fibroblasts, under the influence of various factors, into myofibroblasts [164]. There is also evidence, for lung and liver myofibroblasts, of an origin from a bone-marrow-derived peripheral precursor referred to as a fibrocyte [165, 166]. Other possible origins that have been proposed include epithelial cells which undergo mesenchymal transformation under the effect of the appropriate stressors [167, 168], as well as the pericytes of blood vessels [169].

The stimuli that appear to push fibroblasts to change to myofibroblasts are principally of two kinds – chemical and mechanical.

A number of cytokines have been shown to modulate the fibroblast / myofibroblast population [170]. TGFβ is the most important inducer of myofibroblast differentiation. Its principal mode of action is through the Smad pathway [171], whereby the TGFβ receptor complex promotes binding of Smad-3

with Smad-4 enabling translocation to the nucleus where Smad-3 interacts with the α-smooth muscle actin promoter region. Smad 3-independent αSMA transcription has, however, also been shown in Smad-3 knockout fibroblasts [172].

Apart from cytokines, specialised extracellular matrix proteins appear to be contributors to differentiation. Galectin-3 and fibronectin, more specifically its ED-A splice variant, have been shown to be necessary for TGFβ to have its effect on myofibroblast differentiation [77, 78].

The fibroblasts / myofibroblasts appear to possess mechano-perception – the changes in the stiffness of the surrounding ECM determine the production of αSMA. In fact when fibroblasts are cultured on less elastic surfaces they produce cytoskeletal actin which is not organised into filaments and has only minimal contractile ability. These 'intermediate' cells are termed proto-myofibroblasts [161]. These can then be further pushed into the myofibroblast phenotype by changes in the ECM.

Both fibroblasts and myofibroblasts have extensive contact points between the cytoskeleton and the surrounding matrix. The actin bundle cytoskeleton is associated at its terminal ends with special adhesion sites termed 'fibronexus' in tissue and 'focal adhesions' in cell cultures. The interaction between the actin bundles and the focal adhesions is responsible for transmission of contractile forces generated within the cell to the ECM as well as transmission of forces from outside the cell to the cytoskeleton. A main difference between fibroblasts and myofibroblasts is the change from 'focal adhesions' to 'supermature focal adhesions' which are larger in size and are made up of different proteins. It is hypothesised that with the ongoing remodelling the fibroblast / proto-fibroblast has larger surfaces for adhesion formation, thereby allowing development of stronger stress fibres which in turn can generate stronger contractile forces [173].

A number of biochemical pathways have been linked to changes in mechanical forces acting on the cell [174]. For example, mechanical force has been shown to cause p38 phosphorylation which in turn increases alpha smooth muscle actin expression [175].

Once remodelling has occurred, the mechanical load is now taken over by the extracellular matrix rather than the myofibroblast which then undergoes apoptosis [161, 176]. In disease states, this fails to occur and gives rise to pathological fibrosis [177, 178].

1.7 Cytokines in airway remodelling

A number of mediators may be relevant in the chain of events leading to airway remodelling. These include TGFβ, pro-angiogenic factors such as vascular endothelial growth factor (VEGF), T_H2 cytokines including IL-5, IL-9 and IL-13, Nuclear factor- κB (NF-κB)-regulated chemokines such as Tumour necrosis factor α (TNFα), and various metalloproteases [179]. Three of these mediators – TGFβ, and Galectin-3, two pro-fibrotic agents, and TNFα, a pro-inflammatory agent of particular relevance in severe asthma, have been looked at in this work and are outlined further in the following pages.

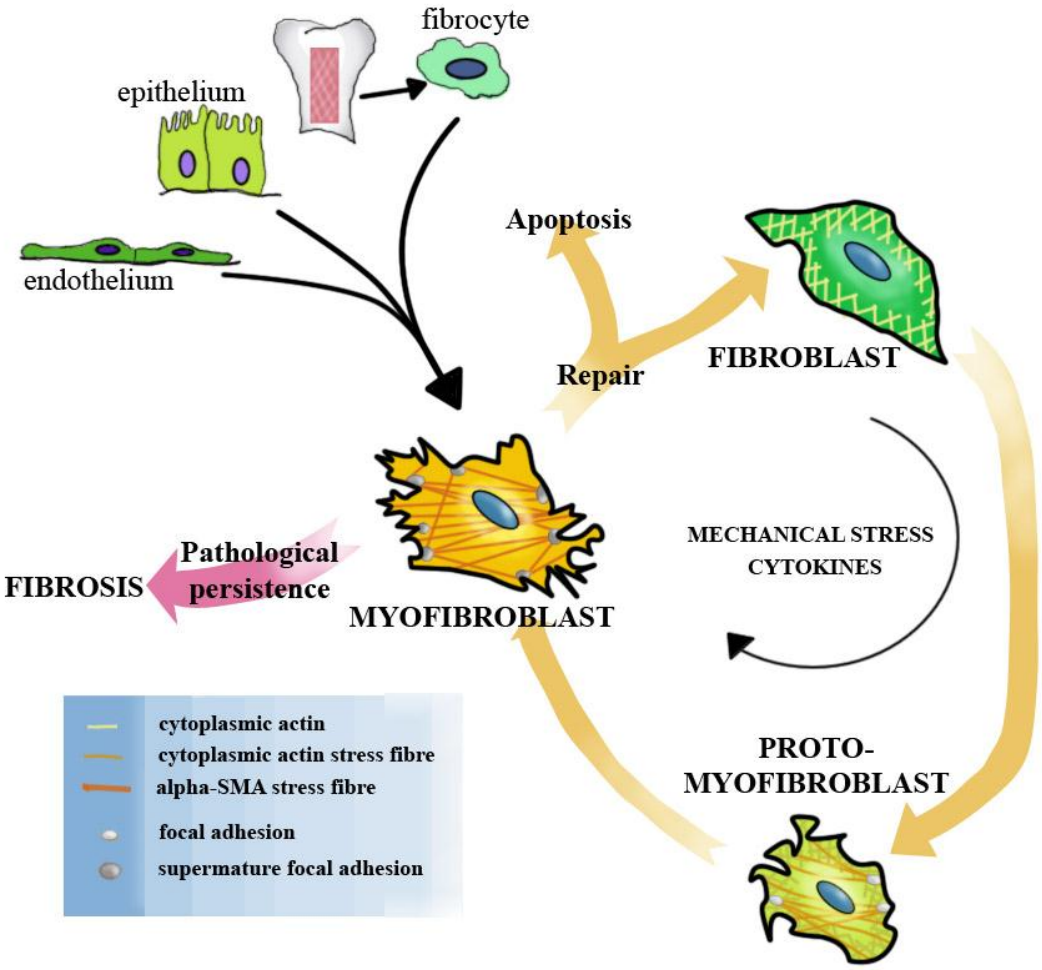

Figure 1.2. The generation of differentiated myofibroblasts. Adapted from [180], [164].

1.7.1 Transforming growth factor beta (TGFβ)

Transforming growth factor beta (TGFβ) is a secreted protein with numerous functions. Five isoforms are described, of which 3 are present in mammals. They are all structurally conserved, have similar functions and act through the same receptor signalling systems. Most cells have the ability to produce TGFβ and have receptors for it. $TGF\beta_1$ is mostly produced by endothelial, haematopoietic and connective tissue cells, $TGF\beta_2$ by epithelial and neuronal cells and $TGF\beta_3$ is expressed mainly in mesenchymal cells [181]. Functions include cell development, growth and differentiation, immunoregulation, cell adhesion and migration and homeostasis of the extracellular matrix.

The active protein is made up of 112 amino acids initially linked with an N-terminal latency associated protein (LAP) which is proteolytically cleaved in the Golgi apparatus. Two TGFβ molecules form a homodimer by binding via a disulphide bond. After interacting with the LAP and latent TGFβ-binding protein (LTBP), the resulting large latent complex (LCC) is then secreted. The LCC then binds to the extracellular matrix until cleavage from the LTBP and the LAP releases the active TGFβ [182].

TGFβ is expressed in the airways and is involved in airway remodelling in various ways. It promotes production of extracellular matrix proteins by fibroblasts as well as decreasing production of collagenases and increasing synthesis of various tissue inhibitors of metalloproteinase [179, 183]. It also promotes airway smooth muscle proliferation [184].

1.7.2 Tumour necrosis factor alpha (TNFα)

Tumour necrosis factor alpha (TNFα) is another pleiotropic cytokine which is mostly associated with inflammation, where it has a role in the regulation of immune cells. Within the airways, it is mostly produced by macrophages but also

by most other pro-inflammatory cells as well as structural cells including epithelial cells and fibroblasts.

It is produced as a membrane-bound 26kD precursor protein, which is released by cleavage of its cytoplasmic tail through the action of TNFα converting enzyme (TACE). The active 17kD protein forms homotrimers which can act on TNFα receptors 1 and 2 expressed on most cells. Its signalling pathway involves upregulation of the transcription factor NF-κB which promotes the transcription of other pro-inflammatory cytokines [185].

As a pro-inflammatory agent, TNFα activates T cells [186] and promotes inflammatory cell recruitment by acting as a chemoattractant for neutrophils and eosinophils [187], as well as by increasing epithelial expression of adhesion molecules [188]. It can increase airway hyperresponsiveness via a number of mechanisms, including the accumulation of pro-inflammatory cytokines within the airway wall as well as direct augmentation of smooth muscle cell contractile function [189]. Its role in airway remodelling is less clear, but the presence of increased concentrations in the bronchoalveolar lavage fluid and bronchial walls of severe refractory asthmatics [190], makes the possibility attractive. It promotes expression of TGFβ [191] and therefore proliferation and differentiation of myofibroblasts, as well as the proliferation of myocytes [192].

1.7.3 Galectin-3

Galectin-3 is a member of a family of galactoside-binding lectins with diverse functions in the regulation of cell proliferation, immunity and tumorigenesis. It is a ubiquitous glycoprotein particularly prevalent in epithelia and inflammatory cells. A number of roles have been postulated for it in asthma including cell recruitment and promoting transformation of fibroblasts to myofibroblasts in

conjunction with TGFβ. A more in-depth overview of its biology and functions is given in Chapter 6.

1.8 Extracellular matrix

The extracellular matrix is an important constituent of the airway wall. This network of macromolecules filling the space between cells, is not just an inert supporting scaffold, but can actively interact with cells of the airway. There are reciprocal chemical and mechanical interactions between the cells and their surrounding matrix which can determine physical properties of the tissue, cell migration, signalling, survival, development, proliferation and function.

The extracellular matrix is made up of two main types of macromolecules: (1) fibrous proteins, such as the collagens, and (2) glycosaminoglycans, usually found bound to protein cores forming proteoglycans [193]. Although precise mechanisms remain to be elucidated, a number of differences in the extracellular matrix have been identified between asthmatics and non-asthmatics.

Fibroblasts / myofibroblasts can secrete a number of components of the extracellular matrix. They have been shown to synthesise collagens, particularly collagens 1, 3 and 5 as well as a number of proteoglycans [194].

The main studies looking at collagen expression in asthma present conflicting evidence. Subepithelial fibrosis is principally made up of collagens 1, 3 and 5 as well as fibronectin and tenascin. Some studies have correlated asthma severity with collagen deposition [107, 195] whilst others have shown no significant relationship [129].

Proteoglycans consist of glycosaminoglycan chains covalently bound to a protein core. Within the extracellular matrix, they cover a number of functions including determination of tissue mechanics, regulation of water balance, cell adhesion,

migration and proliferation and determining biological activities of matrix-bound growth factors and cytokines [196].

A number of proteoglycans appear to be increased in asthma, including versican, biglycan, hyaluronan, perlecan and lumican. As is the case with the collagens, a direct relationship with asthma remodelling and severity has proved more difficult to establish [131].

Further details on collagen and proteoglycan biology are provided in Chapters 3 and 5 respectively.

1.9 Airway wall thickness

An increase in airway wall thickness is one of the described features of remodelling. A number of models have been proposed in order to try to explain the link between changes in airway wall thickness and the symptoms of asthma. The observation that the airways of asthmatics are thicker has been a long established fact from post-mortem observations [105, 197]. However linking this fact with the clinical indices is more complicated.

It is hypothesised that thickening of the submucosa renders it incompressible and therefore makes it impinge more on the airway for any equivalent smooth muscle contraction. This is combined with increased thickness of the adventitial layer which impairs effective transmission of the parenchymal recoil force and results in exaggerated airway narrowing [198]. The increased smooth muscle mass in the airway wall is capable of generating more tension and therefore more narrowing by virtue of its increased bulk and increased contractility in asthmatics [199, 200].

Information about airway wall thickness was initially obtained from post-mortem studies and endobronchial biopsies. Some of these changes are probably transient, related to oedema and cell infiltration or the effect of acute mechanical

strain induced by a fatal asthma attack or a bronchoscopy. Changes, related to matrix deposition and increased muscle mass are probably more permanent. Unfortunately the information that can be obtained ex-vivo is hampered by the artefacts introduced during sample processing, particularly with such elusive elements as wall oedema. This prompted a need to find ways of visualising the dimensions of the bronchial wall in situ. The most common modality to make up for this requirement has been high resolution computed tomography (HRCT) [143, 201-207].

Recently there has been interest in endobronchial ultrasound as a possible tool for assessing airway wall thickness. It has been shown to be as good as HRCT without the associated radiation risk [208] as well as providing the possibility of discerning the various layers of the wall.

Surprisingly, conflicting results have been obtained from different studies looking at airway wall thickness and both baseline FEV_1 and bronchial hyperresponsiveness [143, 201, 206, 209]. One reason for this lack of concordance may be because HRCT can only provide a composite measure of total wall area and changes in the dimensions of the wall components may be more important.

There is remarkably little data in the literature linking airway wall thickness with changes at a molecular level – there is some evidence that it correlates with increased tissue inhibitor of metalloproteinase-1 (TIMP-1) levels in sputum [210] and reticular basement membrane thickness [207].

1.10 Fixed airflow obstruction

Spirometric alternatives for identify remodelling, due to ease of use and diffuse availability, have also been sought in asthma. Fixed airflow obstruction refers to

an obstructive spirometric defect which is irreversible by either bronchodilation and/or suppression of inflammation, and therefore presumed to be structural in nature. A subgroup of asthmatics with an irreversible airflow defect has been identified and characterised in a few studies [211]. As a group they tend to be older, have had asthma for longer and tend to be less atopic. Their asthma is overall more severe with an increased incidence of near fatal attacks [212]. Followed over a period of 5 years, they showed an increased rate of decline of FEV_1 and increased exacerbation frequency [213]. The parameters and cut-off points by which an obstructive defect is defined vary between different studies. Bumbacea *et al* [214] compared two subgroups of severe asthmatics, with a post-bronchodilator FEV_1 of less than 50% or more than 80%, for structural differences using HRCT. Using a radiological scoring system rather than direct measurements, they showed that the intrapulmonary bronchi of the asthmatics with fixed airway obstruction had increased bronchial wall thickening and bronchial dilatation.

1.11 Hypotheses and Aims

Airway remodelling is part of the pathological changes of asthma and to a limited degree is probably a useful protective mechanism from excessive bronchospasm. It is, however, reasonable to assume that once an element of irreversible airflow impairment is introduced, remodelling has over-stepped into clinical significance. It is not clear whether the fixed airflow obstruction is a simple effect of increased airway wall thickness or some other altered physical characteristics of the airway wall. If fixed airway obstruction does equate with measurable airway wall changes, it would be interesting to study this group of severe asthmatics and determine if remodelling is in some way different.

The hypotheses that will be explored in the following work are:

1. Asthmatics with objective airflow defects have more airway remodelling which will be manifested by increased wall thickness.
2. Their fibroblasts will have a different extracellular matrix protein synthetic profile, either at baseline or in response to pro-fibrogenic or pro-inflammatory cytokines.
3. Signalling from damaged epithelium to underlying mesenchymal cells is an important driver of remodelling. One such candidate mediator, galectin-3, may play a role in promoting fibrosis. Galectin-3 is upregulated in the epithelium in asthma, particularly the more severe phenotypes.

CHAPTER 2

Methods

2.1 Subject recruitment

2.1.1 Recruitment methods

Ethical approval for all recruitment was obtained via the Southampton & South West Hampshire LREC.

Subjects for the study were recruited from two main sources.

Volunteers with the appropriate characteristics were selected from the AIR (Allergy and Inflammation Research) division database. These would have submitted their details either personally or online via a volunteer recruitment form. On being identified, they would typically be contacted by telephone and a brief outline of the study explained. If they expressed interest, more detailed written information would be forwarded to them either by e-mail or postal mail. Those who wished to take part would then contact the research unit to agree on dates for the various visits of the study. These constituted the majority of our healthy volunteers and milder asthmatics.

Some asthmatics were approached in clinic, particularly the Southampton University Hospital Trust Difficult Airways clinic, where severe poorly controlled asthmatics are reviewed on a regular basis. Interested subjects would also be given written information and ample time to arrive at a decision as to whether they would like to participate in the study.

A few of the subjects would have decided to take part in the study after hearing about it from someone who had already volunteered.

2.1.2 Inclusion and exclusion criteria

All the subjects for the study had to be between the ages of 17 and 75 years.

Asthmatics had to have at least a one year history of physician-diagnosed asthma.

Non-asthmatics had to report no previous diagnosis of asthma or history suggestive of asthma (no episodes of wheezing, prolonged colds or unexplained cough).

Pregnant subjects or those with significant co-morbidities were excluded. Current smokers or those with a history of more than 10 pack years of tobacco consumption were also excluded.

2.2 Asthma characterisation

2.2.1 History

A thorough history was taken from each subject in order to determine the characteristics of their asthma as best possible.

The salient features of the details collected from each subject are shown in Table 2-1.

Table 2-1. Main points in history taken from volunteers.

Current asthma status -
Frequency of daytime symptoms. Frequency of nocturnal symptoms. Frequency of use of reliever medications. Number of exacerbations in previous 12 months. Number of courses of oral corticosteroids prescribed in previous 12 months. Number of A&E or unscheduled GP visits in previous 12 months. Number of hospitalisations due to asthma in previous 12 months. Current asthma treatment.
Features of past asthma -
Age at diagnosis of asthma. Average yearly number of exacerbations. Average yearly dose of oral corticosteroids taken. Average yearly number of A&E or unscheduled GP visits. Average yearly number of hospitalisations due to asthma. Total number of Intensive Care Unit admissions due to asthma. Past asthma treatment.
Other questions -
Presence of family history of asthma or atopy. Personal history of atopy - allergic rhinitis, atopic dermatitis. Cigarette smoking history. Significant co-morbidities. Concurrent non-asthma medications.

2.2.1.1 GINA classification

Asthma severity was classified according to the Global Initiative for Asthma (GINA) guidelines which are based on symptoms and airflow limitation.

This classification is derived from the older 2005 GINA report (Table 2-2). The more recent report of 2007 [1] advocates a classification based on adequacy of asthma control, which, while more relevant in a clinic scenario, was felt to be less helpful when classifying study subjects.

Table 2-2. Classification of asthma severity by clinical features before treatment.

Step 1: Intermittent
Symptoms less than once a week Brief exacerbations Nocturnal symptoms not more than twice a month FEV_1 or PEF ≥ 80% predicted PEF or FEV_1 variability < 20%
Step 2: Mild persistent
Symptoms more than once a week but less than once a day Exacerbations may affect activity and sleep Nocturnal symptoms more than twice a month FEV_1 or PEF ≥ 80% predicted PEF or FEV_1 variability 20 – 30%
Step 3: Moderate persistent
Symptoms daily Exacerbations may affect activity and sleep Nocturnal symptoms more than once a week Daily use of inhaled short-acting β_2-agonist FEV_1 or PEF 60 – 80% predicted PEF or FEV_1 variability > 30%
Step 4: Severe persistent
Symptoms daily Frequent exacerbations Frequent nocturnal asthma symptoms Limitation of physical activities FEV_1 or PEF ≤ 60% predicted PEF or FEV_1 variability > 30%

Since the majority of our subjects were already on treatment, this had to be taken into consideration when deciding on classification. The recommended method of classification for subjects on treatment is shown in Table 2-3.

Table 2-3. Classification of asthma severity by daily medication regimen and response to treatment.

Symptoms / Lung function	Current treatment step		
	Intermittent	Mild persistent	Moderate persistent
	β_2-agonist as needed	β_2-agonist as needed Low-dose inhaled corticosteroid (or sustained release theophylline, cromone or leukotriene modifier)	β_2-agonist as needed Low-dose inhaled corticosteroid plus long-acting β_2-agonist OR sustained release theophylline or leukotriene modifier OR High-dose inhaled corticosteroid
Step 1: Intermittent Symptoms <1 a week Brief exacerbations Nocturnal symptoms >2 a month Normal lung function between episodes	Intermittent	Mild persistent	Moderate persistent
Step 2: Mild persistent Symptoms >1 a week but <1 a day Nocturnal symptoms >2 a month but <1 a week Normal lung function between episodes	Mild persistent	Moderate persistent	Severe persistent
Step 3: Moderate persistent Symptoms daily Exacerbations may affect activity and sleep Nocturnal symptoms at least 1 a week 60% < FEV_1 < 80% predicted OR 60% < PEF < 80% personal best	Moderate persistent	Severe persistent	Severe persistent
Step 4: Severe persistent Symptoms daily Frequent exacerbations Frequent nocturnal asthma symptoms FEV_1 ≤ 60% predicted OR PEF ≤ 60% personal best	Severe persistent	Severe persistent	Severe persistent

Using the information on symptom control and current treatment, the asthmatic subjects were classified into intermittent, mild persistent, moderate persistent and severe persistent asthma.

Subject classification and characteristics are outlined in the relevant chapters.

2.2.1.2 Issues in asthma classification

There are various problems in trying to classify a heterogeneous disease like asthma into well-defined categories. The GINA classification, which encompasses both symptoms and airflow limitation to assess severity was felt to be best suited for this study.

However, since the classification is based on the worst symptom or parameter, it is necessarily dependent on the volunteers' subjective experience of their disease which can vary a lot and may not tally with physiological parameters. Even putting aside the issue of symptom subjectivity, since many of our severe asthmatics were recruited from a tertiary referral clinic, there is a definite bias towards refractory asthma in this group. This may mean that there may be true steroid-resistant asthmatics or subjects with other factors which may be contributing to their symptoms but are not directly arising from airway inflammation, such as vocal cord dysfunction, gastro-oesophageal reflux or post-nasal drip. On the other hand, a realistic classification with all its limitations is more encouraging when translating any findings to the real world clinic scenario [215-219].

In order to further ensure categorization into more markedly differing groups, the intermittent, mild and moderate persistent asthma subjects were clustered together as "mild" asthma.

FEV_1, reversibility, PEF variability values and ACQ scores showed significant difference between the healthy or milder asthmatics and the severe ones, but not between healthy and mild asthma subjects in our subject cohorts.

2.2.1.3 ACQ score

Each subject filled in an Asthma Control Questionnaire (Table 2-4). This is a validated questionnaire consisting of six questions regarding symptoms over the previous week and reliever medication use. That day's percent predicted FEV_1 makes up the seventh parameter. All variables are scored on a 7-point scale (0 to 6 in increasing severity) [220]. The six questions were answered by the volunteer whilst the measurement of FEV_1 was performed later in the day. Each answer has equal weighting – the final score being calculated as a mean of the 7 individual scores [220, 221]. It has been validated for distinguishing well-controlled and poorly-controlled asthma although correlation with lung function is more equivocal [222].

2.2.2 Physical examination

A full physical examination was performed to exclude any co-morbidities not identified in the interview as well as to obtain baseline parameters.

Table 2-4. Asthma Control Questionnaire.

Circle the number of the response that best describes how you have been during the past week.			
Question 1. On average, during the past week, how often were you woken by your asthma during the night?	0 Never 1 Hardly ever 2 A few times 3 Several times 4 Many times 5 A great many times 6 Unable to sleep because of asthma	Question 4. In general, during the past week, how much shortness of breath did you experience because of your asthma?	0 None 1 A very little 2 A little 3 A moderate amount 4 Quite a lot 5 A great deal 6 A very great deal
Question 2. On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?	0 No symptoms 1 Very mild symptoms 2 Mild symptoms 3 Moderate symptoms 4 Quite severe symptoms 5 Severe symptoms 6 Very severe symptoms	Question 5. In general, during the past week, how much of the time did you wheeze?	0 Not at all 1 Hardly any of the time 2 A little of the time 3 A moderate amount of the time 4 A lot of the time 5 Most of the time 6 All the time
Question 3. In general, during the past week, how limited were you in your activities because of your asthma?	0 Not limited at all 1 Very slightly limited 2 Slightly limited 3 Moderately limited 4 Very limited 5 Extremely limited 6 Totally limited	Question 6. On average, during the past week, how many puffs of short-acting bronchodilator (e.g. Ventolin) have you used each day?	0 None 1 1-2 puffs most days 2 3-4 puffs most days 3 5-8 puffs most days 4 9-12 puffs most days 5 13-16 puffs most days 6 More than 16 puffs most days
Question 7. To be completed by a member of the clinic staff.			
Record actual values on the dotted lines and score the FEV_1 % predicted in the next column	FEV_1 pre-bronchodilator FEV_1 predicted FEV_1 % predicted	....	0 >95% predicted 1 95-90% 2 89-80% 3 79-70% 4 69-60% 5 59-50% 6 <50% predicted

2.2.3 Spirometry

2.2.3.1 General principles

Spirometry was performed using a Vitalograph wedge bellow spirometer. The Vitalograph is a type of dry-bellows spirometer which is particularly suitable for measuring rapid changes in volume. It is widely used and has been shown to give reliable measurements [223] of forced expiratory volumes and forced expiratory flow rates, which are important in the diagnosis and management of asthma. It has a built-in motorised carrier for graph paper and a pen which is driven by the bellows [224], giving a volume-time plot.

Two basic readings are obtained – the forced expiratory volume in 1 second (FEV_1) which is the volume of air expelled in the first second of a forced expiration and the forced vital capacity (FVC) which is the total volume of air exhaled in a forced expiratory manoeuvre.

The FEV_1 is easily obtained and quite reproducible [225]. For the most part it is an effort-independent measure, although it does depend on the subject performing a proper maximal inspiratory flow manoeuvre. It has poor sensitivity, partly because it mostly reflects large airway obstruction and therefore gives no indication of the increased peripheral resistance that is present in some asthmatics despite a normal FEV_1 [136].

A correlation with mortality from chronic airways disease has been shown [226, 227], but there is a less clear relationship with asthma severity [228, 229].

The FVC is mostly used to derive the ratio of FEV_1 to FVC which reflects airflow limitation. FVC decreases with airway collapse where dynamic compression of the airways during a forced expiration traps air which is therefore not expelled. It, in turn, can increase following therapy or recovery from an exacerbation. All this

makes the use of FEV_1/FVC a less useful index of obstruction than FEV_1 on its own [230].

2.2.3.2 Methodology

Spirometry was performed according to published guidelines [231, 232].

Asthmatics were asked to refrain from using a long-acting β_2-agonist for at least 12 hours and short-acting β_2-agonist for at least 6 hours prior to the procedure.

The subject was asked to sit down a comfortable distance away from the device. The procedure was explained and a clip was applied to the nose. The subject would take a deep breath inwards trying to fill up the lungs to maximum capacity, and then seal the lips tightly around the mouthpiece and blow forcefully outwards. They were encouraged to keep on exhaling actively until all air was expelled to achieve forced vital capacity.

Attempts were repeated (up to a maximum of 8 or until the patient was too exhausted to continue) until 3 readings within 10% of each other were obtained.

The best absolute value was recorded as well as the calculated percent predicted. Percent predicted values were obtained from European Coal and Steel Community spirometric reference values [233]. These particular set of reference values are widely used in our department although there is some evidence to suggest that they underestimate both FEV_1 and FVC [234]. Unfortunately no set of reference values is without problems [235].

2.2.4 Reversibility

2.2.4.1 General principles

Reversibility of airflow obstruction is tested by comparing FEV_1 values before and after administration of a β_2-agonist. Healthy individuals have been shown to have a mean increase in post-bronchodilator FEV_1 of 2.5% [236, 237]. Reversibility is considered to be present if the FEV_1 increases by at least 12% from baseline and 200 ml in volume. This eliminates changes due to variability of spirometric readings [238] and is higher than the reported post-bronchodilator response in healthy populations [239, 240].

There is no clear consensus as to the optimal dose of β_2-agonist that should be used nor the ideal time interval to wait after administration to check for reversibility [241, 242]. The ATS/ERS task force for standardisation of lung function testing recommends using 400µg salbutamol or albuterol and rechecking spirometry between 10 and 15 minutes later [231]. Use of a spacer is recommended to ensure a less technique-dependent delivery of drug to the lung [243].

It is also unclear as to which parameter best expresses reversibility although the percentage change in FEV_1 appears to be the most useful in distinguishing asthma from other obstructive lung diseases and from non-asthmatics [244].

Reversibility is affected by baseline FEV_1 – the lower it is the higher the potential reversibility [245]. There is also a slight increase in reversibility with age and female gender [246]. Surprisingly, severe asthmatics with non-reversible airflow limitation have actually been shown to have similar reversibility values to those with less fixed impairment [214].

The test has a high false-negative rate – the presence of a high reversibility makes asthma as the cause of the airflow limitation very likely but the opposite may not be true [247].

2.2.4.2 Methodology

Reversibility was assessed after administration of 400µg salbutamol metered dose inhaler via a volumatic spacer. Spirometry was performed, in the same way as baseline assessment, 15 minutes after administration of bronchodilator. The reversibility was assessed as percentage change in FEV_1 from the pre-bronchodilator reading.

2.2.5 Skin prick testing

2.2.5.1 General principles

Skin prick testing was used to identify IgE-mediated hypersensitivity to aeroallergens. It involves exposing dermal mast cells to antigens via epicutaneous administration to identify if they express cell surface specific IgE. If so, this results in histamine release leading to oedema and erythema, giving the classical wheal-and-flare skin lesion [248].

Allergic sensitisation varies widely in different communities [249], as does the range of aeroallergens [250] but it is a known risk factor for asthma [251-253], especially in children [254, 255]. The prevalence of asthma increases with the number of positive skin prick tests in children [256] and is a predictor of disease persistence [257].

2.2.5.2 Methodology

Skin prick testing was performed in all subjects. A panel of aeroallergens obtained via GA^{2}LEN (Global Allergy and Asthma European Network) was used. This

consisted of – pollen allergens (Birch, Alder, Hazel, Plane, Cypress, Olive, Grass mix), mould allergens (Alternaria, Cladosporium, Aspergillus), weed allergens (Artemisia, Ambrosia, Parietaria), animal allergens (Cat and Dog), the house dust mite allergens (Dermatophagoides pteronyssinus, Dermatophagoides farinae), and Cockroach (Blatonella).

Skin prick testing was performed by applying a small drop of each of the above 18 allergens, as well as a positive control (histamine) and a negative control (allergen diluent), on the flexor aspects of the subject's forearms. The subjects were asked to refrain from use of antihistamines for at least 4 days prior to the test. The skin was then pricked with a sterile lancet, changed for each allergen, in order to allow allergen penetration into the subcutis. After 15 minutes, the size of any resulting wheals was measured. Two measurements were recorded in millimetres – the largest diameter of the wheal and the diameter perpendicular to it. An average of the two was considered to be positive if more than or equal to 3mm greater than the saline negative control [258].

2.2.6 Exhaled Nitric Oxide

2.2.6.1 General principles

Nitric oxide (NO) is thought to participate in a number of physiological processes including vasodilatation, neurotransmission, bronchodilation and host defence. It is produced in a number of cells by the action of the enzyme nitric oxide synthase from L-arginine [259-261]. The presence of measurable NO in the exhaled breath of humans was first described in 1991 [262].

In asthmatic airway inflammation there is upregulation of inducible nitric oxide synthase within the epithelium [260] which leads to additional NO generation above what is found in non-asthmatic airways [263-267]. Correlations between

exhaled NO levels and induced sputum eosinophilia [268] as well as bronchial hyperresponsiveness have been described [269].

There are a number of potential confounding factors. Atopy on its own may give higher readings in children [270] and adults [271] whilst treatment with inhaled corticosteroids gives readings comparable to non-asthmatics [264-266, 272, 273].

In spite of this, exhaled NO may have a role in asthma diagnosis [274], monitoring of anti-inflammatory treatment response [275] and compliance [276] as well as predicting impending exacerbations [277].

Most analysers of nitric oxide use chemiluminescence to quantify the gas. This method involves the reaction of NO with ozone in order to produce nitrous oxide (NO_2) and oxygen. This NO_2 is in an energized state and a small fraction of it decays to baseline and emits a photon in the process. By measuring the photons emitted in the near infra-red portion of the spectrum, the concentration of NO can be determined [278, 279].

There are differences in the values of NO obtained depending on the analyser used [280], the method of calibration [281], as well as the airflow generated by the subject [282].

A significant amount of NO is derived from the upper respiratory tract particularly the sinuses [283], therefore measures are adopted to exclude this during the procedure [284].

Dupont *et al* [285], using a chemiluminescence analyser, determined that a cut-off threshold of 16 parts per billion (ppb) gave a 90% specificity and a positive predictive value of over 90% in distinguishing steroid-naive mild asthmatics from healthy subjects.

2.2.6.2 Methodology

Exhaled nitric oxide was measured using a Logan Research Trace Gas and Nitric Oxide Analyser LR2500 (Logan Research, Rochester, Kent, UK). The machine was calibrated on the day of use with NO at a known specific concentration. After the test was explained to the subjects, they would be asked to sit down and put on a nose clip. The test involves taking a deep breath to full inspiration and then blowing into the machine's mouthpiece maintaining as constant a flow as possible. An LED display helps the subject to gauge flow adequately. A real-time display of the NO level is shown on a monitor. The output typically shows an initial peak representing washout of NO from upper airways or nasal NO if velum is not closed during the early phase of exhalation, followed by a plateau [286]. The subject would be asked to continue exhalation for a maximum of 20 seconds or less if they get tired. The level of NO was measured at the plateau. Two readings within 10% of each other were obtained and their mean calculated and recorded.

2.2.7 Methacholine bronchial challenge

2.2.7.1 General Principles

Airway responsiveness to non-sensitising stimuli such as methacholine is measured to determine the tendency of the airway to bronchoconstrict in response to non-specific stimuli.

There are a number of methods used to measure airway responsiveness. They are broadly divided into direct and indirect methods. Direct methods make use of a targeted stimulus on the effector cell – usually the airway smooth muscle cell, but also to some degree the endothelial and mucus-producing cells, to bring about airflow limitation. Indirect methods act through mediator release from

inflammatory cells, neuronal cells and epithelial cells which will then signal the effector cells.

These two methods are thought to be measuring different components of airway hyperresponsiveness. Indirect stimuli, such as adenosine are shedding light on the contribution of airway inflammation to the heightened bronchial response. This tends to fluctuate more, depending on the prevalent allergen exposure of the subject, level of asthma control and treatment administered. On the other hand direct methods are presumed to reflect the more permanent airway changes that arise in some asthmatics [287].

Methacholine is a synthetic analogue of acetylcholine which stimulates muscarinic post-ganglionic parasympathetic receptors to bring about smooth muscle contraction [288].

The increased responsiveness of asthmatic airways to non-specific stimuli including methacholine can be attributed to various factors. The smooth muscle layer in asthma shows hypertrophy and hyperplasia as well as increased contractility. It is also hypothesised that a reduced baseline airway calibre could mean that any smooth muscle contraction results in a relatively larger reduction in airflow [287].

The test involves exposing the subject to sequential doubling doses of nebulised methacholine and checking for a drop in FEV_1 from baseline at each step. A 20% drop in FEV_1 has been determined as a meaningful fall and is used in determining airway hyperresponsiveness as it can be achieved in asthmatics but is often not achievable in healthy subjects [289]. The concentration of methacholine that brings about this drop is denoted the provocative concentration causing a 20% drop in FEV_1 (PC_{20}).

With methacholine, a relationship between the degree of responsiveness and symptoms has been shown. In a study by Hargreave *et al*, all asthmatics with recent symptoms had a PC_{20} less than 8 mg/ml whilst all non-asthmatics had a PC_{20} of more than 8 mg/ml. Although there is a lot of overlap [290], bronchial challenge has been repeatedly shown to be one of the more reliable tests in asthma diagnosis [247, 291].

Increased airway responsiveness is associated with more severe asthma in children [292], increased peak flow variability and good response to bronchodilators [293].

A number of confounding issues can affect the test results. A degree of increased airway responsiveness is demonstrable in all obstructive airways diseases, not just asthma [294], as well as in allergic rhinitis [295]. There is also a lot of variation in values depending on the population being studied [296].

A proportion of the general population has asymptomatic hyperresponsiveness [297, 298]. These subjects have been shown to have a degree of airway inflammation intermediate between normal subjects and asthmatics [299] and over time have an increased risk of developing asthma symptoms [300, 301].

2.2.7.2 Methodology

Methacholine challenge was performed according to published guidelines [302].

Methacholine (Provocholine 100mg, Methapharm) was serially diluted in the 24 hours preceding the test. Serial dilutions in 0.9% saline between 0.03 mg/ml and 16 mg/ml were made up in separate tubes.

The procedure was explained to the subject. A baseline FEV_1 and FVC were recorded. If the baseline FEV_1 was less than 60% of predicted, the bronchial challenge was not performed.

Serial nebulisations were performed via a Spira elektro2 inhalation dosimeter (Respiratory Care Center, Hanneenlina, Finland). The subject was asked to take 5 inhalations via the dosimeter with a nose clip in place. To ensure adequacy of each inhalation, an LED display which lights up with airflow was used. Best of three FEV_1 readings were recorded at 1 minute and 3 minutes post-each inhalation. The lower of the two values was then used in calculations.

The first nebulisation was with the diluent on its own – in this case 0.9% saline. A drop in FEV_1 of 10% or more from baseline would represent a significant drop with the sole diluent and the test was discontinued. After this, further nebulisation with methacholine was done, starting at 0.03 mg/ml and recording FEV_1 at 1 minute and 3 minutes each time. Further nebulisations at 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8 and 16 mg/ml were performed. The test was discontinued when either a 20% or higher drop from post-saline FEV_1 was registered or the maximum dose was reached without a diagnostic drop. The concentration inducing a 20% fall was determined from the concentration response curve.

2.2.8 Peak flow diary

2.2.8.1 General principles

Peak expiratory flow (PEF) is the largest flow rate achieved with maximal expiratory effort from maximum inspiration. It is usually attained within the first 100ms of forced expiration. It is effort-dependent and is affected by respiratory muscle strength, subject motivation and airway calibre [303]. Assuming the first two remain constant, PEF reflects airway calibre.

FEV_1 and PEF are both meant to be indices of airway calibre, but although there is a high degree of correlation between the two [304] they do not tend to be equivalent – FEV_1 tending to be lower when expressed as a percentage of predicted than PEF [305, 306]. Since PEF is measured during the early part of

expiration it reflects large airway calibre whilst FEV_1 is also determined by medium-sized airways.

Regular PEF monitoring over a 24 hour period reveals a circadian rhythm of recorded values in all subjects. This peak flow variability is significantly higher in asthmatics [307].

Since the readings are effort-dependent, performed while unsupervised and usually need to be done more than once daily over a period of weeks there is a lot of concern regarding patient compliance and the reliability of readings [308].

Under study conditions, repeatability of readings has been shown to be good in both healthy volunteers and asthmatics [309, 310].

Peak flow variability values correlate to some degree with airway hyperresponsiveness [293, 311, 312] although this is not substantiated in all studies [313]. Regular use of corticosteroids results in improvement in mean PEF and reduced PEF fluctuation [314], presumably secondary to control of airway inflammation [315].

We opted to use the mini-Wright peak flow meter, the most commonly used device for ambulatory peak flow measurement in the UK. This works on the basis of airflow causing movement of a piston linked to a tension spring – the distance covered depending on the flow rate [316]. It has been shown to be simple to use and reliable [317].

Although peak flow measurement has a well-established role in the management of asthma there is no standardised method of quantifying PEF records.

One of the big issues with the use of peak flow data is how best to summarise the data in order to reflect airflow variability [303, 318].

A number of methods for measuring diurnal variation are used in the literature. The more commonly used ones are outlined in Table 2-5.

Table 2-5. Peak flow variability measures in common use.

Average amplitude	Average [highest PEF on the day – lowest PEF on the day]
Amplitude percent highest	Average [(highest PEF on the day – lowest PEF on the day) / highest PEF on the day * 100]
Amplitude percent mean	Average [(highest PEF on the day – lowest PEF on the day) / period mean PEF * 100]
Morning dip	Average [(morning PEF / highest PEF of the day) * 100]

Other methods described involve more extensive calculations and tend to be less commonly used (Table 2-6).

Table 2-6. Less commonly used peak flow variability measures.

Standard deviation percent mean	(Standard deviation of PEFs / period mean PEF) * 100
Coefficient of variation	(Period mean PEF / Standard deviation)
Cosinor amplitude percent mean	(Amplitude of cosinor wave (constructed by computer software) / mean of cosinor wave) * 100

Few studies have directly compared the different summarisation strategies. Reddel *et al* [319] compared various measures of morning dip (period lowest or average morning PEF expressed as a proportion of recent best or predicted PEF), amplitude percent highest and mean PEF as a proportion of standard deviation or predicted PEF. They found good correlation of these indices with airway hyperresponsiveness, and to a lesser degree with symptom scores and airway obstruction – the highest correlation being registered with minimum morning PEF percent of recent best or predicted PEF.

Higgins *et al* [320] compared average amplitude, amplitude percent highest, amplitude percent mean, standard deviation of PEFs, standard deviation percent mean PEF and cosinor amplitude percent mean to identify the best discriminator between asthma and non-asthma. Amplitude percent mean and standard deviation percent mean provided the best option, the former being recommended as it is simpler to calculate.

2.2.8.2 Methodology

Subjects were given a new peak flow meter if they did not have one or they were asked to use their own if it was less than a year old [321].

They were shown how to perform peak expiratory flow rate measurement using a mini-Wright peak flow meter (Clement Clarke international, Harlow, Essex, UK) at the first visit. The manoeuvre was performed standing up with a forceful expiration performed after full inspiration with the lips tightly sealed around a disposable cardboard mouth piece. Three readings were performed and the highest value achieved recorded. Supervised peak flows were performed before and after salbutamol as part of the work up in the first visit. This enabled identification and correction of any deficiencies in their technique.

They were then asked to record their peak flows in the morning and evening, ideally before use of bronchodilators, in the two weeks immediately preceding the bronchoscopy.

On the same peak flow chart they were also asked to record their symptoms, on a 0 to 6 scale of severity. With the morning PEF, they would document the preceding night's symptoms whilst the daytime symptoms were recorded with the evening PEF. They also recorded the frequency of use of reliever inhaler medications used over those 24 hours.

Period PEF variability was then calculated adopting the period amplitude percent highest method, which is most commonly used in our clinics. In our subjects, this was found to have good correlation with the amplitude percent which has shown good specificity in asthma [320].

$$\text{Percentage PEF period variability} = \left(\frac{\text{Highest period PEF} - \text{Lowest period PEF}}{\text{Highest period PEF}}\right) \times 100$$

2.3 Bronchoscopy

Bronchoscopy was performed according to published guidelines [322, 323]. The safety of this procedure has been demonstrated even in severe asthmatics, provided the necessary precautions are taken [324-327].

The procedure was explained to the subjects during study consent taking and re-outlined on the actual bronchoscopy day. Baseline readings were taken prior to the procedure including blood pressure, pulse, pulse oximetry and an FEV_1. Females of a child bearing age had a urine pregnancy test to exclude pregnancy. The volunteers, both asthmatics and non-asthmatics, were administered 2.5mg of nebulised salbutamol to counter-act any possible bronchospasm during the subsequent procedure.

Local anaesthetic (lignocaine) was applied via spray to the back of the throat followed by intravenous atropine 500mcg and titrated intravenous sedation with midazolam and/or fentanyl. The bronchoscope was passed through the nostril or mouth, through the vocal cords and into the large airways. Local anaesthetic was applied through the scope as it was introduced into the various areas of the bronchial tree.

Oxygen saturation was monitored during the procedure using a pulse oximeter, and supplemental oxygen applied via nasal cannulae as necessary.

Endobronchial ultrasound was performed as outlined below.

Bronchoalveolar lavage, bronchial brushings and endobronchial biopsies were obtained at endoscopy for each subject, where possible.

At the end of the procedure, the subject was allowed to rest for an hour, after which an attempt was made at oral intake by asking them to sip on some water to ensure satisfactory swallowing co-ordination. Subjects were discharged after ensuring FEV_1 was no longer showing a significant drop in volume from the pre-bronchoscopy value.

2.3.1 Bronchoalveolar lavage

Bronchoalveolar lavage was performed by wedging the scope in a segmental bronchus of the right upper lobe, introducing warmed 0.9% saline via 6 aliquots each containing 20 ml and then aspirating it 10 seconds after each instillation.

After retrieval, the lavage was passed through a 100µm filter under gravity then spun at 2000RPM for 10 minutes. The supernatant was divided into aliquots and stored at -80°C until used. Cells were re-suspended and a cytospin made.

2.3.2 Bronchial brushings

Brushings were usually obtained from the right main bronchus using 2mm-channel disposable cytology brushes (Olympus Medical Systems Corp, Tokyo, Japan). The brush was inserted through the working channel of the endoscope, with the bristles enveloped in a sheath. Once in the desired bronchus, the brush end was unsheathed and under direct vision was scraped along a part of the bronchial wall to collect superficial bronchial wall cells. The brush end was re-sheathed and removed from the scope. The cells were then transferred to containers with phosphate-buffered saline (PBS) and subsequently used for cell

culture. Some brushings were transferred to containers with Trizol™ (phenol and guanidine isothiocyanate solution, Invitrogen, Paisley, UK) for subsequent RNA (ribonucleic acid) extraction.

2.3.3 Bronchial biopsies

Bronchial biopsies were performed at the segmental carinae of the right lower lobe using Precisor disposable 1.8mm alligator cup biopsy forceps (ConMed Endoscopic Technologies, Swindon, UK). The closed forceps were passed through the bronchoscope's working channel, and then used to bite on the chosen segmental or sub-segmental carina. Biopsies were obtained by giving a sharp tug to the biopsy forceps and then pulling it out of the bronchoscope. The contents of the biopsy forceps cup were inspected to ensure that there was a reasonably sized biopsy and then transferred to the appropriate medium for immunohistochemistry processing or Trizol™ extraction.

2.4 Endobronchial ultrasound

2.4.1 General principles

Ultrasound imaging involves the use of high frequency waves (>20, 000Hz) to delineate tissues of different acoustic impedance. The waves are created by applying an alternating current to a piezoelectric crystal which then vibrates at a specific frequency which is transmitted directly to adjacent tissues. The wave propagates by molecular displacement. When it encounters a tissue with different acoustic impedance, some of it will proceed through the second tissue, some will undergo refraction and some will be reflected back (Figure 2.1). The piezoelectric crystal itself can convert the incoming reflected vibrations to electrical impulses which can then be built into an image – distances being calculated from time and velocity of the reflected wave. There is a progressive energy dissipation of the

wave through friction, as well as refraction of waves which fail to make it back to the receiver. This progressive loss of signal, referred to as attenuation, is proportional to the frequency – the higher the frequency the more significant the attenuation and therefore the lower the tissue penetration. On the other hand, a higher frequency gives a better image resolution due to the shorter wavelength (distance between peaks of successive waves). A compromise therefore has to be made between depth penetration and image resolution.

The acoustic impedance of a tissue is determined by its density and the propagation velocity of the ultrasound through that particular tissue. The latter is in turn dependent on the particle mass (which also determines density) and the elastic forces binding the particles together. The bigger the difference in acoustic impedance at an interface between two tissues the more reflected waves are created, and consequently less waves progress further. In the case of endobronchial ultrasound, this means that a transducer positioned in the middle of a bronchus would be useless as most of the ultrasound wave would be reflected at the bronchial wall. By interposing a fluid interphase between the transducer and the tissue, this difference is attenuated and usable images obtained – a process referred to as sonic coupling.

Apart from post-mortem studies, the most commonly used way of assessing airway wall thickness has been by high resolution computed tomography [143, 201-207]. Endobronchial ultrasound was initially studied as a tool in assessing bronchial wall tumour invasiveness [328]. Shaw *et al* have shown it is as good as high resolution computerised tomography in measuring airway wall thickness in asthma without the associated radiation risk [208].

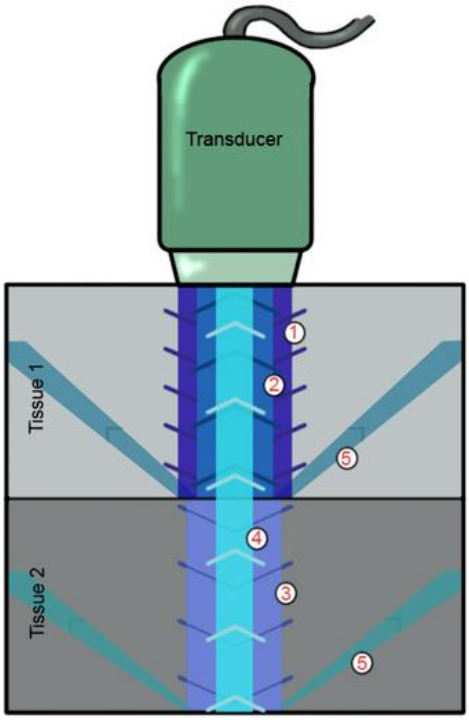

Figure 2.1. General principles of ultrasound.

Ultrasonic waves (1) are generated at the transducer and travel through tissue. At a boundary between tissues of different acoustic impedance, some waves (2) will be reflected back to the transducer. A proportion (3) will propagate through to the adjacent tissue. At the next boundary, further waves (4) are reflected back. An image is generated from the reflected waves. Progressive loss of energy occurs through friction as well as waves that are reflected away from the transducer (5).

2.4.2 Methodology

Endobronchial ultrasonography was performed during the bronchoscopy. This was performed prior to any of the other procedures as its main aim was to assess bronchial wall dimensions which could potentially be affected by lavage, brushings or biopsies.

The radial ultrasound probe was introduced into a specially made sterile balloon sheath. This was pre-filled with sterile water, ensuring no air bubbles remained trapped within the inflatable balloon at the tip.

During the procedure, the scope was introduced into the distal bronchus intermedius of the right lower lobe. The ultrasound probe, within its sheath, was

then introduced through the working channel of the scope and advanced towards the posterior basal segmental bronchus (B^9). The balloon was then inflated, just outside the orifice of B^9 (Figure 2.2) until a satisfactory image of the airway was achieved. A video recording of the images from start of inflation to complete deflation was stored on the ultrasound machine. Where possible, more than one inflation-deflation was performed for each subject.

At a later date, the videos were reviewed and the best still images for each subject retrieved. The image selected was the one in which the airway wall was acceptably viewed with the least balloon inflation, and therefore least wall distortion.

The chosen images were then transferred to a personal computer, anonymised and image analysis and measurements were performed in a blinded fashion using computer software (Osiris Medical Imaging software v 4.19, University of Geneva, Switzerland) [329].

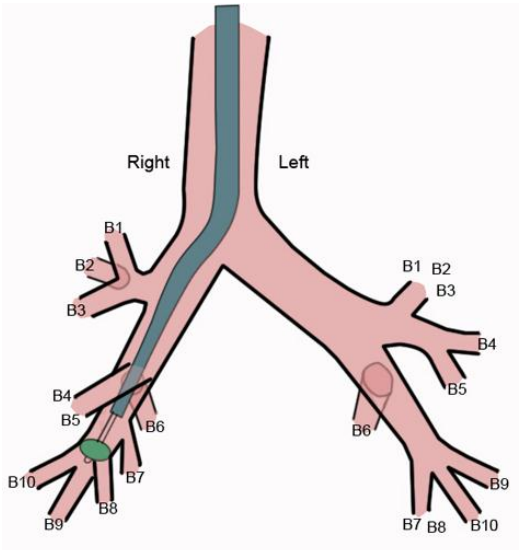

Figure 2.2. Schematic diagram of bronchial anatomy with site of balloon inflation for ultrasound imaging.

2.5 Immunohistochemistry

2.5.1 GMA embedding

2.5.1.1 General Principles

Immunohistochemistry involves the use of labelled antibodies against specific antigens within tissues or cells to localize and quantitate them.

A very important consideration is the preservation of antibody-binding sites from the point in time when the tissue or cell is still alive until it is exposed to the antibody of choice. This epitope preservation has to occur in the presence of morphological preservation.

Embedding in glycol methacrylate (GMA) optimally enables conservation of tissue morphology with an acceptable level of preservation of antigenic epitopes. It has particular applicability for small tissue samples as is the case with bronchial biopsies [330].

2.5.1.2 Methodology

Bronchial biopsies were retrieved at bronchoscopy and transferred immediately into ice cold acetone (Fisher scientific, UK Cat no. A/0600/25) containing protease inhibitors – 2 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, UK Cat no. P7626) and 20 mM iodoacetamide (Sigma-Aldrich, UK Cat no. I6125). They were left to fix overnight at -20°C.

The next day the fixative was replaced by dry acetone at room temperature for 15 minutes and subsequently by methylbenzoate (Fisher scientific, UK Cat no. M/4460/PB08) for a further 15 minutes. After this, the biopsies were put in 5%

methyl benzoate in GMA monomer solution A at 4°C for 3 incubations of 2 hours each.

For the final stage, the biopsies were transferred to the GMA embedding resin solution (JB4 embedding kit, Park Scientific, Northampton, UK Cat no. 00226), consisting of GMA monomer solution A, GMA catalyst solution B and benzoyl peroxide. The biopsy was placed at the bottom of a labelled TAAB flat-bottomed capsule (Taab, Aldermaston, UK Cat no. 0094) which was then filled to the brim with the embedding solution. This was left to polymerise for 2 days at 4°C and subsequently stored at -20°C in airtight containers containing silica gel until used for immunohistochemistry.

2.5.2 Cutting GMA biopsies

When ready for staining, the GMA biopsies were retrieved from storage and removed from the plastic capsules. Excess GMA was removed using a file, approximating the borders to the embedded biopsy. Two micrometre sections were cut sequentially from the biopsy using a glass knife microtome (Jung Supercut 2065, Leica, UK). The cut biopsies were placed on 0.01% poly-L-lysine (Sigma-Aldrich, UK Cat no. P8920) coated glass slides (Knittel Glaser, Braunschweig, Germany) – typically two sections per slide. If immunostaining was not performed on the day of cutting, the slides were stored at 4°C and used within a week.

2.5.3 Immunostaining

2.5.3.1 General principles

The addition of antibody-antigen interactions to histochemical techniques enables the identification of patterns of distribution of a large number of antigens.

These techniques have evolved from direct immunofluorescence, which involves a single step fluorescently labelled antibody, to indirect methods with two or three-tiers of interactions (Figure 2.3). More interactions allow magnification of binding sites which increases the sensitivity of the assay.

Primary antibodies are produced via a process involving injection of the antigen being investigated into a specific animal species to elicit antibody production. Polyclonal antibodies generated in this way can be extracted from the animal's serum. In the case of monoclonal antibodies, B cells from the animal are fused with myeloma cells to produce a hybridoma. The hybridomas producing the best antibody are selected, cloned and monoclonal antibodies harvested from them [331]. Most antibodies are commercially available.

Before applying the primary antibody the tissue is first swamped with a blocking serum containing a vast range of immunoglobulins in order to minimise non-specific binding of this antibody to other epitopes, to which it might have some cross-affinity. The blocking medium, coming from a non-immunised organism, will not specifically recognise the target antigen but will tend to block a large number of epitopes that tend to bind antibodies non-specifically.

The primary antibody is then applied to the tissue. This will bind to the target antigen. Excess antibody is washed off and a secondary antibody is applied. This is, usually, a polyclonal antibody against the Fc fragment of the immunoglobulins of the animal species to which the primary antibody belongs. By virtue of its polyclonality, it binds to the primary antibody in a proportion of at least two to one. The secondary antibody is bound to biotin, a low molecular weight vitamin.

The third stage of the process involves a complex of avidin or streptavidin with biotin and horseradish peroxidase. Avidin is a glycoprotein derived from egg albumin which has 4 high-affinity binding sites for biotin per molecule. Streptavidin, a related protein derived from *Streptomyces avidii*, is usually

preferred as it has no carbohydrate-binding sites which can give high background staining from binding to lectins in the tissue. Horseradish peroxidase is bound to biotin, which in turn binds to streptavidin. Large molecules can be created in this way allowing many peroxidase molecules to link to one secondary antibody. Mixing proportions must be calculated to ensure some free binding sites on the streptavidin to allow interaction with the biotin on the secondary antibody.

Detection of this three-tiered system occurs through the interaction of a chromogen with horse-radish peroxidase, which catalyses a reaction resulting in a colour change. Since most tissues are rich in endogenous peroxidase which is abundantly found in peroxisomes and macrophages, this has to be neutralised, usually by adding its natural substrate, hydrogen peroxide, in the early stages of the process. The chromogens most often used are diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) which give a brown and red colour reaction respectively [332].

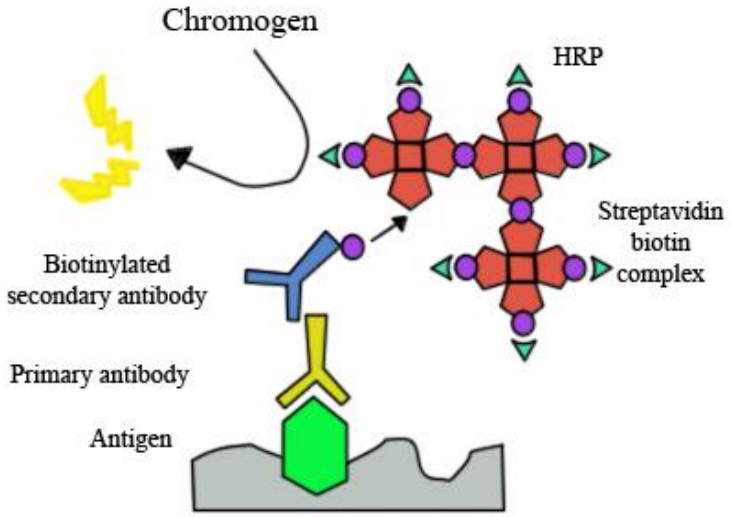

Figure 2.3. Basic schematic of three-tiered system used in immunohistochemical staining.

2.5.3.2 Methodology

The following protocol was used for immunostaining of bronchial biopsies.

Slides were placed on staining trays and allowed to reach room temperature if they had been stored in the cold. Endogenous peroxidase was inhibited using a solution of 0.1% sodium azide (Fisher Scientific, UK Cat no. S/2380/48) and 0.3% H_2O_2 (Sigma-Aldrich, UK Cat no. H1009) applied to each biopsy for 30 minutes at room temperature. Slides were then washed with Tris Buffered Saline (TBS) 3 times, incubating them at room temperature for 5 minutes each time. TBS is a solution of 150mM Sodium Chloride (Fisher Scientific, UK Cat no. S/3120/60), 50mM Tris (Tris (hydroxymethyl) methylamine 'Tris buffer', Fisher Scientific, UK Cat no. T/P630/60) and 1M Hydrochloric acid (5M HCl, Fisher Scientific, UK Cat no. J/4310/15) adjusted to a pH of 7.65. After draining excess TBS, they were then incubated with blocking media (Dulbecco's Modified Eagle Medium or DMEM with 20% FBS and BSA) for 30 minutes at room temperature. Primary antibodies, as shown in Table 2-7, were prepared at the appropriate dilutions in TBS determined by previous titration experiments. Isotype control staining is performed for each antibody on a separate occasion, to ensure that there is no non-specific binding.

Table 2-7. Primary antibodies used in immunohistochemistry.

Antigen	Supplier	Type	Chromogen	Dilution
Galectin 3 [9C4]	Abcam	Monoclonal mouse	DAB	1 : 100
Collagen 1 [COL-1]	Abcam	Monoclonal mouse	DAB	1 : 500
Collagen 3 [3G4]	Chemicon	Monoclonal mouse	DAB	1 : 1500

After the primary antibodies were applied to the biopsies, the slides were protected with cover slips (Menzel-Glaser, Germany) and incubated overnight at room temperature. In the case of controls, TBS was applied to the slide instead.

The following day, the cover slips were removed and the slides washed 3 times with TBS as described in a previous step.

The secondary anti-species antibody – biotinylated polyclonal rabbit anti-mouse $F(ab')_2$ antibody (Dako, UK Cat no. E0413) – was prepared at the appropriate dilution in TBS. After draining the slides, the stage 2 antibody was put on the biopsies and incubated for 2 hours at room temperature. Preparation of the streptavidin-biotin-horse radish peroxidase labelled (StABC-HRP) complex (StABC-HRP complex, Dako, UK Cat no. K0377) was done at least 30 minutes before it was due to be applied on to the slides. This involved mixing streptavidin and biotinylated horse radish peroxidase according to the manufacturer's instructions. Washing of slides with TBS was performed 3 times for 5 minutes each, as previous. Once the slides were drained, the labelled StABC-HRP complex was pipetted onto the biopsies and kept there for 2 hours at room temperature. The slides were again washed 3 times with TBS and drained.

The chosen chromogen was made up as per manufacturer's instructions – either AEC (AEC substrate pack, Launch Diagnostics, UK Cat no. HK129/5K) or DAB (Liquid DAB substrate pack, Launch Diagnostics, UK Cat no. HK153/5K) and added to the biopsies, for 20 minutes in the case of the former and for 10 minutes in the case of the latter. They were then rinsed with TBS and submerged in running tap water for 5 minutes. Counterstaining was performed with Mayer's haematoxylin and blue for 90 seconds to outline tissue morphology. The slides were immersed for a further 5 minutes in running tap water, then drained. Aqueous mounting medium (AbD Serotec, UK Cat no. HIS002C) was applied to the slides which were then dried in an oven at 80°C for 10 minutes. After cooling, the slides were mounted in Pertex (Surgipath, UK Cat no. 08706E) with cover slips. Staining was quantified using computer-assisted image analysis software (Zeiss KS 400 image analysis system, Welyn Garden City, UK).

2.6 Fibroblast culture

Primary fibroblasts were grown from bronchial wall biopsies obtained at bronchoscopy.

Routine culture of fibroblasts was performed in Dulbecco Modified Eagle's Medium (DMEM + 4.5g/l glucose, Gibco Invitrogen, UK Cat no. 11960-044) with 10% (v/v) heat-inactivated foetal bovine serum (FBS, Sigma, UK, Cat no. F-3018), 1% Sodium Pyruvate (100mM, Gibco Invitrogen, UK Cat no. 11360-039), 1mM Non-essential amino acids (100x MEM NEAA, Gibco Invitrogen, UK Cat no. 11140-035), 50 IU/ml Penicillin and 50 µg/ml Streptomycin (PenStrep, 5000 units/ml penicillin and 5000 mcg/ml streptomycin, Gibco Invitrogen, UK Cat no. 15070-063) and L-Glutamine (100x L-Glutamine 200mM, Gibco Invitrogen, UK Cat no. 25030-024).

All procedures were performed in a laminar flow hood.

Fibroblast culture involved four types of procedures: (1) initial growing of primary fibroblasts from the biopsy, (2) splitting or passaging the fibroblasts when they reach confluence, (3) cryopreserving the fibroblasts in liquid nitrogen and (4) regrowing cryopreserved cells.

2.6.1 Growing fibroblasts from biopsy

The following protocol was used to encourage fibroblasts to grow out of the biopsy.

At bronchoscopy the biopsy (typically 0.5 – 1mm diameter) was placed in a 'bijou' container (7 ml sterile polystyrene bijou with cap, Fisher Scientific, UK Cat no. DIS-080-010R) filled with supplemented Dulbecco Modified Eagle's Medium, as above. The biopsy was kept in this medium on ice until it could be processed

further – typically within 60 minutes of retrieval at bronchoscopy. Clots and mucus were removed from the biopsy by successively transferring it through two containers with the same growth medium using a pastette (Alpha laboratories, UK Cat no. LW4232).

The biopsy was then placed in one well of a 6-well plate (Tissue culture Nunclon flat bottom tissue culture polystyrene radiation sterilised 6 round well plate, Fisher Scientific, UK Cat no. TKT-190-110E). Using a scalpel and blade (Swann-Morton, Sheffield, UK), the biopsy was scored into the plastic of the bottom of the well. This allows the biopsy to be divided into small fragments which get embedded into the well bottom.

About 3 ml of the same supplemented DMEM was then added to the well. The remaining 5 wells of the plate were filled with sterile phosphate buffered saline (PBS) to minimise drying.

Medium was changed daily until the fibroblasts could be seen growing out of the biopsy. After this, medium changes were done three times weekly using supplemented DMEM.

2.6.2 Passaging of fibroblasts

The following protocol was used to 'passage' or split the fibroblasts once they reached confluence in the 6-well plate.

When the fibroblasts reached sub-confluence within the well, the medium was removed. Three millilitres of Hank's balanced saline solution without calcium or magnesium (HBSS, Gibco Invitrogen, UK Cat no. 14170-088) pre-warmed to 37°C was added to the well taking care not to disrupt the cell layer. This was swirled around to wash the cell layer thoroughly from any residual medium. The HBSS was then removed and a second HBSS wash was repeated in the same way.

In order to detach the cells, 2 ml of Trypsin/EDTA (0.5% 10X Trypsin/EDTA, Gibco Invitrogen, UK Cat no. 15400-054) diluted to 1X concentrate in HBSS and pre-warmed to 37°C was applied to the well covering the entire well bottom and then removed. The plate was placed in the incubator for about 60 seconds for the protease to work.

About 3 ml pre-warmed supplemented DMEM were added to the well, pipetted up and down repeatedly to help in dislodging cells and then transferred to a 50 ml-conical bottom centrifuge tube (Greiner, UK Cat no. 210261). The well was rinsed with a further 3 ml complete DMEM which was then added to the centrifuge tube.

A further 4 ml DMEM was added to the cell suspension which was then transferred into a new 75cm^2-flask (Tissue culture flask Nunc EasY Flask angled neck polystyrene radiation sterilised filter cap 75cm^2 growth area, Fisher Scientific, UK Cat no. TKT-130-210T).

The cells were kept in an incubator, changing medium three times a week until 90% confluence was reached.

The following protocol was used to 'passage' or split the fibroblasts once they reached confluence in the 75cm^2-flask.

The medium was removed from the flask. About 10 ml of pre-warmed HBSS without calcium or magnesium was added, swirled around and then removed. The HBSS wash was repeated.

Cells were dislodged by adding 2 ml pre-warmed Trypsin/EDTA into the flask. It was swirled around to cover the bottom of the flask and then removed. The flask was placed in the incubator for about 60 seconds and was subsequently inspected under the microscope and tapped sharply to dislodge all the cells.

The Trypsin was neutralised by addition of 5 ml pre-warmed supplemented DMEM. The trypsinised cells were then transferred to a 50 ml-conical bottom centrifuge tube. A further 5 ml complete DMEM was added to the empty flask swirled around and pipetted into the centrifuge tube to maximise retrieval of cells. When necessary, a cell count was performed at this stage. The cells were then transferred into a new 75cm^2-flask which was then placed in the incubator.

At passage 3, a sample of the fibroblasts was transferred to a chamber slide and stained for α-smooth muscle actin and vimentin using fluorescein-tagged antibodies. Manual counts of a representative area of the slide were made to determine proportion of αSMA-positive cells. A proportion of less than 10% was considered to be acceptable – if the percentage was higher the cells were not used for experiments.

In routine cell culture, cells were cryopreserved at each passage, ensuring a minimum of $0.75x10^6$ cells per cryovial and a minimum of $0.2x10^6$ cells for the new flask.

2.6.3 Cryopreserving fibroblasts

Three cryovials were stored whenever possible at each passage, between the second and fifth passage. The following protocol was used for freezing down the cells in liquid nitrogen.

The initial steps followed were as for 'passaging' of fibroblasts up to the application and neutralisation of Trypsin. After trypsinisation, a cell count was performed and a volume of the cell suspension containing a minimum of $0.2x10^6$ cells was transferred to a new 75cm^2-flask rather than being centrifuged with the rest.

The remaining cell suspension was centrifuged at 1500RPM at 25°C for 5mins. After centrifugation, the medium was tipped off and the cells re-suspended.

Three millilitres of Dimethyl Sulphoxide (DMSO Hybri-Max, Sigma-Aldrich, UK Cat no. D2650) 10% (v/v) in DMEM previously prepared and chilled were added to the cell suspension.

The cells were then split equally into a number of cryovials, ensuring a minimum of 0.75×10^6 cells per cryovial. The cryovials were wrapped in tissue paper, placed in a plastic bag and put in the -80°C freezer overnight before being transferred to liquid nitrogen on the following day.

2.6.4 Reconstitution of cryopreserved fibroblasts

The following protocol was used to reconstitute cryopreserved fibroblasts.

The cryovial was retrieved from liquid nitrogen storage. Supplemented DMEM (pre-warmed to 37°C) was added to the cryovial, thawing the cell suspension relatively quickly. This was transferred to a $25cm^2$-flask (Tissue culture flask Nunc EasY Flask angled neck polystyrene radiation sterilised filter cap $25cm^2$ growth area, Fisher Scientific, UK). Further supplemented DMEM was added to the cryovial to ensure maximal retrieval of cells, and transferred to the flask.

A further 3ml DMEM was added to the flask, in order to dilute the potentially cytotoxic DMSO. The flask was placed in an incubator for 4 hours, to allow the reanimated fibroblasts to attach to the bottom. After the four hours, the medium was changed removing non-viable cells as well as any residual DMSO.

The cells were then kept in the incubator with 3-weekly changes of medium until 90% confluence was reached. They would then typically be passaged to one or more $75cm^2$-flasks, depending on cell count and experimental requirements.

2.7 Enzyme-linked immunosorbent assay

2.7.1 General Principles

Direct and sandwich enzyme-linked immunosorbent assays (ELISAs) were used in this project. The basic process involves antigen-antibody binding which is detected through an enzyme catalysed substrate colour change. Quantification is achieved by running a standard curve on known concentrations of the antigenic protein.

In a sandwich ELISA, special high-capacity protein-binding ELISA plates are coated with an antibody specific to the antigen of interest. After washing off unbound antibody, any vacant binding sites are blocked with protein. The samples, as well as negative controls, are then added to the wells. A number of wells are filled with doubling concentrations of the antigen to obtain the standard curve. Any antigen present in standards, samples or controls will bind to the capture antibody. A secondary antibody bound with enzyme is added after washing off unbound antigen. This is designed to bind to a different epitope of the antigen. In the streptavidin-biotin system, the secondary antibody is bound with biotin, which will then bind with the enzyme, horse radish peroxidase in the subsequent step. This step allows an amplification to occur in a fashion similar to what has been outlined for immunohistochemistry above. Finally, the substrate on which the enzyme will act is put into the wells. The resultant colour change is measured via an absorbance plate reader. By comparing the absorbance data from the samples with those obtained for the known concentrations in the standard curve, the presence of antigen in the samples can be quantified [333].

In the direct ELISA, the antigen is already bound to the plate wells and there is no secondary antibody. The primary antibody, which is already biotinylated, is added to the wells and binds to the antigen on the wells. The streptavidin-biotin

complexes will then bind directly to the primary antibody and bring about a colour change on being exposed to the substrate.

2.7.2 Methodology

Different variations of ELISA were used – the specific methods are outlined in the respective Results chapters.

2.8 RNA Extraction

2.8.1 General Principles

RNA extraction was performed using the Trizol™ method. This is based on the single-step process first described by Chomczynski and Sacchi [334]. Trizol™ is a mono-phasic solution of acidified phenol and guanidine thiocyanate. This is used to lyse the tissue or cells from which the RNA needs to be extracted. It disrupts the cell components but maintains the integrity of the nucleic acids – mostly through the action of the isothiocyanate which eliminates enzyme activity especially that of RNases. Chloroform is then added and the RNA separated out by differential solubilisation. The acidification of the phenol keeps the DNA (deoxyribonucleic acid) in the organic phase whilst the RNA dissolves in the water component. The sample is centrifuged and separates into three phases – a lower red organic phase and an upper aqueous phase separated by a milky interphase. The RNA is retrieved by pipetting out the transparent aqueous phase. The RNA is then precipitated out of solution by addition of isopropanol.

When the RNA is being used for PCR, it is very important to eliminate any genomic DNA which can give false readings. Treatment with DNase is performed after RNA precipitation to degrade any genomic DNA.

2.8.2 Methodology

The whole process was performed in a clean working area. This is very important to minimise as much as possible the effects of RNases which can degrade RNA and therefore reduce the yield of the extraction. Before starting, the working area was sprayed with RNase-zap (Sigma-Aldrich, UK, Cat no. R2020) then washed with RNase-free water (MilliQ, Millipore, UK). Disposable gloves were worn at all stages to avoid contamination with skin commensal organisms which may contain RNases. All pipette tips were DNase-RNase-free ART tips (Aerosol Resistant Tips, Molecular BioProducts, San Diego, US). All microtubes used were DNase/RNase-free microfuge tubes (Sigma-Aldrich, UK, Cat no. T3566).

2.8.2.1 RNA precipitation part 1

RNA samples were obtained from cells grown in monolayers or bronchial brushings. Cells were lysed by adding 1 ml of Trizol™ (Trizol reagent, Invitrogen, UK Cat no. 15596-018) to the well, leaving it for around 5 minutes and then pipetting it a few times up and down before transferring it to a DNase/RNase-free microfuge tube. The samples were stored at -20°C until further extraction was performed.

Samples were thawed and 200µl chloroform added per 1 ml Trizol™. The samples were then shaken vigorously by hand and incubated at room temperature for 10 minutes. This dissociates any nucleoprotein complexes that may have formed.

The microfuge tubes with Trizol™ samples and chloroform were centrifuged at 12 000 RPM for 15 minutes at 4°C. Spinning needs to be done at a low temperature to minimise the quantity of DNA that may sequester into the aqueous phase.

The samples will separate into a transparent top layer containing the RNA, a cloudy interphase containing mostly DNA and a pink phenol phase containing the protein.

The top aqueous layer was transferred using a pipette into a fresh RNA microfuge tube being careful not to transfer any of the other phases.

When RNA was being extracted from small quantities of cells (10^2–10^4 cells) or tissue (1–10mg) 5–10µg RNase-free glycogen (Glycogen from mussels, Sigma, UK, Cat no. G1767) was added per 500µl sample. Thorough mixing was assured by vortexing the samples.

RNA precipitation was then achieved by adding 500µl isopropanol (Sigma, UK, Cat no. 19516) per tube and mixing well by shaking.

The samples were stored overnight at -20°C to increase precipitation yield.

2.8.2.2 RNA precipitation part 2

Samples were thawed, vortexed and then incubated at room temperature for 15 minutes and vortexed again.

They were then centrifuged at 12 000 g for 30 minutes at 4°C to obtain a pellet of precipitated RNA.

The supernatant was tipped off, taking care not to disrupt or lose the pellet.

The remaining isopropanol was washed off with 75% ethanol – made up by diluting 100% ethanol (Riedel-de Haën, Germany Cat no. 32221) with DNase/RNase-free water. One ml 75% ethanol was added to each sample which was then centrifuged at 7500 g for 5 minutes at 4°C.

The ethanol was poured off. It is important to remove ethanol thoroughly as it may inhibit many downstream applications. In order to ensure this, the samples were pulse spun to clear sides of ethanol. Any remaining ethanol was carefully removed with a P20 pipette, paying attention not to let ethanol drip down the side of the tube.

Samples were air-dried for 5-10 minutes. This gets rid of any residual ethanol – but it is important not to over-dry the pellet as this makes re-suspension very problematic.

2.8.2.3 DNase treatment

DNase treatment removes trace contamination by genomic DNA.

A DNase master mix consisting of the DNase enzyme (rDNase I, Ambion, US, Cat no. 2224G), a buffer (10x DNase I buffer, Ambion, US, Cat no. 8169G2), which ensures optimal conditions for the enzyme activity, and water was prepared (Table 2-8).

Table 2-8. DNase master mix.

Reagent	Volume
DNase enzyme	1µl
10X Buffer	2µl
DNase/RNase-free water	17µl

The constituents were mixed up in a microfuge tube, the volumes calculated according to the number of samples treated. Twenty µl were added to each RNA sample.

The tubes were incubated for 1 hour at 37°C, giving the enzyme the necessary time to work.

The DNase action is stopped by addition of an inactivator (DNase inactivation reagent, Ambion, US, Cat no. 8174G) – 5µl was added to each sample, mixed thoroughly and incubated for 2 minutes at room temperature.

The samples were pulse spun for 2 minutes and stored at -80°C until reverse transcription was performed.

2.9 Reverse Transcription

2.9.1 General Principles

Reverse transcription is the process by which the extracted RNA is transcribed into complementary DNA (cDNA) through the use of an RNA-dependent DNA polymerase (commonly referred to as a reverse transcriptase). Reverse transcriptases are found in a certain class of RNA viruses, the retroviridae which use this enzyme to change their RNA into DNA which can then be incorporated into the host genome to be translated into the virus proteins.

The technique involves two steps – the annealing step in which a primer binds to the RNA and the extension step in which the reverse transcriptase adds nucleotides to the primer.

2.9.2 Methodology

The amount of RNA extracted from each sample is calculated using spectrophotometry. This was performed using a Nanodrop spectrophotometer (ND-1000, Nanodrop technologies, US). A 1µl quantity was used to get a measurement of RNA concentration. This particular piece of equipment creates a column of the RNA in solution between two pedestals by surface tension. A spectrophotometer then passes light through the liquid. Readings of absorbance

are recorded at 260nm and 280nm – these are dependent on the presence of different proportions of nucleotides in the RNA and DNA. The ratio of 260nm to 280nm absorbance gives an indication of the purity of RNA, whilst concentration is derived from the 260nm absorbance value. A ratio between 1.8 and 2.0 was deemed to indicate RNA of acceptable purity.

2.9.2.1 Annealing

The reactions were performed in thin-walled microfuge tubes to maximise heat transfer.

The extracted RNA was thawed and pulse spun to separate out the DNase-plus-inactivator slurry.

The annealing mix (Table 2-9) was first made up according to the quantities needed. It includes an Oligo-dT primer which binds to the poly-A 3'-end tail of mRNA and a nucleotide (dNTP) mix.

One µg RNA from the liquid phase of the sample was added to the corresponding labelled thin-walled tube. The volume equivalent to 1µg RNA was calculated from the concentration of RNA determined by spectrophotometry. Oligo-dT primer and dNTP mix was then put in, 2µl per sample, and the final volume made up to 10µl in each thin-walled tube by adding distilled water.

After centrifugation to mix well, the samples were heated in a 'heat block' thermocycler (Eppendorf Mastercycler Gradient) at 65°C for 5 minutes. This denatures the RNA secondary structure. At the end of this period, they were quickly 'snap cooled' by transferring to ice which allows the primer to anneal to the RNA.

Table 2-9. Annealing master mix.

Reagent	Volume
Oligo-dT	1 µl
dNTP mix	1 µl
RNA	x µl – equivalent to 1 µg RNA
RNA / DNase free water	y µl – to make up to 10 µl
ANNEALING MIX – final volume 10 µl	

2.9.2.2 Extension

The extension mixture (Table 2-10) was then made up. This consists of the reverse transcriptase enzyme derived from Moloney murine leukaemia virus (MMLV) that mediates the synthesis of the complementary DNA strand. MMLV Reverse Transcriptase (MMLV RT) lacks a 3' to 5' exonuclease proofreading function and has a weak RNase H activity. RNase H activity selectively degrades the RNA of RNA-DNA hybrids [335] and means that the RNA template is destroyed after first strand complementary DNA synthesis.

The other components of the extension mixture are a buffer to ensure an optimal milieu for reverse transcriptase activity and water to make up the mixture to 10µl per sample.

Ten µl of this mixture was added to each sample, lid applied and samples spun for adequate mixing.

They were then transferred to a water bath preheated to 37°C and floated on it for 15 minutes, after which the temperature was increased to 42°C for 60 minutes.

The resulting cDNA was then stored at -20°C until ready to be used.

Table 2-10. Extension master mix.

Reagent	Volume
5X RT buffer MMLV	4 µl
MMLV RT enzyme	0.8 µl
RNA / DNase free water	5.2 µl
EXTENSION MIX – final volume 10 µl	

2.10 Polymerase chain reaction

2.10.1 General Principles

Polymerase chain reaction (PCR) involves exponential amplification of specific DNA sequences to enable detection and quantification. The process involves denaturation of the double stranded DNA into single strands, in order to permit binding of primers. These primers are short sequences of nucleotides, usually about 20 bases in length that are complementary to a defined sequence on the DNA of interest. Two primers – a 'forward' and a 'reverse' primer are designed to attach to each of the two strands of DNA. Through the action of a DNA polymerase, nucleotides are added at the 3' end of the primer thus extending it and producing a new copy of each DNA strand. By repeatedly cycling this process, doubling of the DNA of interest can be achieved at each step.

PCR was revolutionised in the mid-1980s [336, 337] by the introduction of a thermo-stable DNA polymerase derived from *Thermus aquaticus* (Taq DNA polymerase), a species of bacteria that grows in geysers at temperatures of above 100°C. Since this polymerase does not get inactivated at the temperatures necessary for DNA denaturation – the whole process could be automated.

Apart from the DNA polymerase, the primers and the DNA sample, the PCR reaction requires other factors to work. Magnesium ions (usually in the form of

$MgCl_2$) are a necessary co-factor for the polymerase reaction. In order for extension to occur nucleotides in excess need to be present, as well as a buffer which ensures stability of pH for optimal DNA polymerase activity [338].

A master mix is made up of Taq polymerase, $MgCl_2$ and deoxyribonucleotide triphosphate (dNTP) mix in a reaction buffer to which the primers and the DNA are added.

Each PCR cycle consists of three steps (Figure 2.4):

Denaturation – the DNA is heated to 95°C for around 1 minute to separate the DNA into single strands.

Annealing – binding of the primers is then promoted by reducing the temperature between 45°C and 60°C for another minute. The temperature for this step is calculated as 5°C lower than the melting temperature of the primer-DNA template duplex. One primer will bind to one DNA strand and the other binds to the complementary strand.

Extension – addition of nucleotides occurs through the action of the DNA polymerase at its optimal working temperature of 72°C. DNA synthesis will occur through the region of interest and for variable distances into the flanking region, depending on the distance between the two primers. The duration of the extension step depends on the length of the desired PCR products – usually 1 minute is sufficient to synthesise fragments as long as 2 kilobases.

Repeated cycles, usually between 25 and 45, are programmed and performed automatically within a thermocycler yielding a doubling of the DNA at the end of each cycle and therefore an amplified amount of target PCR product.

The addition of real-time detection methods enhances the capabilities of PCR by making it a quantitative assay. This is based on measuring emission of

fluorescence during the amplification of the DNA which is proportional to the number of amplicons (or replicating DNA fragments) being produced.

Fluorescence can be generated in one of two main ways – agents that bind to double-stranded DNA and fluorescent probes.

In the former instance, agents such as SybrGreen become very fluorescent on binding to double-stranded DNA – the amount of fluorescence increasing with the number of copies of DNA being produced.

Fluorescent probes, on the other hand, are short DNA sequences with a reporter fluorochrome at the 5’ end and a quencher fluorochrome at the 3’end, which effectively neutralise each other. These probes capitalize on the 5' to 3' exonuclease activity of Taq polymerase, which cleaves the internal probe during PCR. With the reporter separated from its quencher, fluorescence is emitted – the amount depending on the number of amplicons.

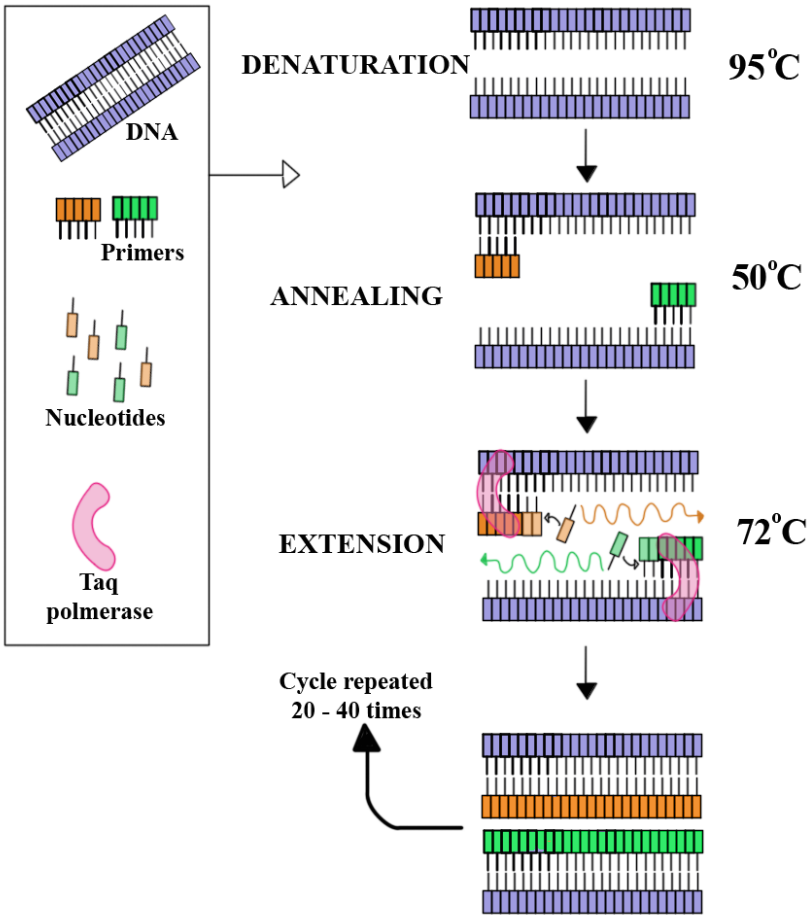

Figure 2.4. The main steps of a polymerase chain reaction.

Fluorescence is monitored in real time and a threshold, during the exponential phase of the amplification when the fluorescence emitted goes above the background signal, is determined. The cycle at which this occurs, the threshold cycle (Ct) depends on the amount of DNA present at the beginning of the reaction. The difference in Ct at which any two different samples cross the threshold

corresponds to the difference in their initial concentration. Relative expression of a gene of interest is calculated by normalising for loading differences in the amount of DNA by determining Ct values for a reference gene (a gene whose expression is known to remain constant under the studied conditions) and then comparing with a calibrator, or control sample.

2.10.2 Methodology

The preparation for a PCR reaction involved planning a plate setup. This includes wells for the cDNA samples in duplicate and negative controls. Wells containing water rather than cDNA and wells with non-reverse-transcribed RNA to exclude the presence of contaminating genomic DNA, were used as negative controls.

A PCR mix was made up with PrecisionTM 2X qPCR Mastermix (PrimerDesign, Southampton, UK) which consists of 2x reaction buffer, 0.025 U/µl Taq Polymerase, 5 mM $MgCl_2$ and dNTP mix (200µM each dNTP). To this was added the primer or primer/probe mix, depending on the assay being used, and then made up to the calculated volume with RNase/DNase-free water. Primer sequences of genes studied are outlined in Table 2-11. The volumes used were calculated according to the number of samples being analysed. The make-up of the PCR mix varied according to the type of assay being used – further details are outlined in the text that follows.

Table 2-11. Sense and anti-sense primer sequences (5' to 3').

Gene	Sense primer	Anti-sense primer
Decorin DCN	GCCACTATCATCCTCCTTCTG	TAGCATAAAGTCAAATAAGCCTCTC
Biglycan BGN	GTCTGAAGTCTGTGCCCAAAG	GCTCGGAGATGTCGTTGTTC
Lumican LUM	AACCACAACAACCTGACAGAG	GTGCTGGAGATGGATGAAGG
Versican VCAN	GGAGAAGTGGATATTGTTGATTCAT	CTTGGCACCTCAGGATGTTT
Galectin-3 LGALS3	AAAGAGGGAATGATGTTGCC	AAATGGGAAAACCGACTGTC

The PCR mix was added to a 96-well PCR plate (iQ 96-well PCR plates, Bio-Rad, California, US Cat No. 223-9441). . After this, the cDNA template, diluted 1:10, was put into the respective wells, 2.5µl per well. RNase/DNase-free water was added to the negative wells and RNA at the appropriate concentration was put into another two wells. The PCR plate was then sealed with an appropriate PCR plate sealer (Microseal 'B' film, Bio-Rad, California, US INN120411).

The plate was pulse spun to ensure complete pooling of the mixture in the wells.

The PCR was performed on an iCycler Thermal Cycler (Bio-Rad, California, US). The protocol used depended on the type of PCR run being performed – the protocols are outlined below.

Three different types of assays were performed.

1. PerfectProbe primer/probe assays (PrimerDesign, Southampton, UK).

PerfectProbes are hairpin hydrolysis probes which combine very close proximity of quencher and reporter dye (as in Molecular Beacon) and hydrolytic probe dissociation through the action of polymerase (as in Taqman). This provides low background fluorescence at the start of the reaction and high endpoint fluorescence.

With this assay, the PCR mix was made up as shown in Table 2-12.

Table 2-12. PCR master mix for PerfectProbe.

Reagent	Volume
PrecisionTM 2X qPCR Mastermix	7.5µl
Primer/probe mix	1µl
RNase/DNase-free water	4µl

The PCR protocol used on the thermal cycler is outlined in Table 2-13.

Table 2-13. PCR protocol for PerfectProbe.

Step	Temperature (°C)	Time (min)	Repeats
Enzyme activation	95	08:00	1
Denaturation	95	00:10	50
Annealing + Data collection	50	00:20	
Extension	72	00:10	

2. Multiplex PerfectProbe assays (PrimerDesign, Southampton, UK).

Multiplex reactions were used when checking for reference genes. In this case the reaction involves using primer/probe mixtures containing two primers and two probes with different fluorochromes for two separate genes. Since there are two fluorochromes which emit fluorescence at different wavelengths, the expression of two different genes can be determined.

The PCR mix used in this assay is shown in Table 2-14. Larger volumes of Mastermix were used due to the need to detect two genes.

Table 2-14. PCR master mix for multiplex PerfectProbe.

Reagent	Volume
PrecisionTM 2X qPCR Mastermix	12.5µl
Primer/probe mix (two genes)	1µl
RNase/DNase-free water	9µl

The PCR protocol used on the thermal cycler is outlined in Table 2-15.

Table 2-15. PCR protocol for PerfectProbe multiplex.

Step	Temperature (°C)	Time (min)	Repeats
Enzyme activation	95	08:00	1
Denaturation	95	00:10	50
Annealing + Data collection	50	00:45	
Extension	72	00:10	

The data collection step is prolonged compared to the standard protocol due to the need to acquire a larger volume of information.

3. SybrGreen assays (PrimerDesign, Southampton, UK).

A commercial master mix containing SybrGreen was used and since the reaction does not depend on fluorescent probes, primers only are added to this PCR mix as shown in Table 2-16.

Table 2-16. PCR master mix for SybrGreen.

Reagent	Volume
PrecisionTM 2X qPCR Mastermix with SybrGreen	7.5µl
Primer	1µl
RNase/DNase-free water	4µl

The PCR protocol used (Table 2-17) has an added melt curve step. Since SybrGreen binds to any segment of double-stranded DNA and emits fluorescence, it is important to ascertain that the amplification curve is the result of one PCR product. At the end of the PCR itself, all products are heated to 95°C to denature them. The temperature is then reduced to 50°C and increased in a stepwise fashion. The melting temperature of any segment of double-stranded DNA depends on its length and the frequency of the stronger Guanine-Cytosine (G-C) bonds within it. So, as the temperature is increased, different double-stranded amplicons will denature thus releasing SybrGreen and resulting in a drop in fluorescence. If more than one amplicon is present, this drop will appear at more than one temperature, and a plot of negative first derivative (-Δfluorescence/Δtemperature) against temperature would have more than one peak.

Table 2-17. PCR protocol for SybrGreen.

Step	Temperature (°C)	Time (min)	Repeats
Enzyme activation	95	08:00	1
Denaturation	95	00:15	45
Annealing + Data collection	60	01:00	
Denaturation	95	03:00	1
Melt curve	Starting at 50°C with 0.5°C increments	00:10	90

2.10.3 Calculations

Calculations of relative gene expression were done using the delta-delta Ct method. At the end of the PCR run, a set of Ct values are obtained for each well – this represents the point at which the fluorescence from that well is higher than background (Figure 2.5). Since samples are analysed in duplicate, an average of the two Ct values is used in calculations. If there is a more than 1-fold difference between the two duplicates, the amplification curves are inspected and the Ct value corresponding to the 'cleanest' curve is used rather than the average.

The lower the Ct value of any sample, the higher the expression of the gene of interest.

In order to make allowance for differences in the concentration of cDNA introduced in each well, the expression of a reference gene is assessed by PCR. Reference genes are chosen according to the cell type being studied. They have to be adequately expressed in the tissue of interest and show minimal variability in expression between samples and under the experimental conditions used. This corrects for differences in amount of tissue sampled, and differences in RNA extraction and reverse transcription efficiencies. In most instances more than one reference gene expression is measured for each sample.

For each sample, the subtraction of the geometric mean of the Ct values for the reference genes from the Ct value for the gene of interest gives the ΔCt. By then comparing the expression of the gene of interest in various samples with a calibrator – usually untreated or normal samples, the ΔΔCt is derived. The fold-difference is then obtained by calculating $2^{-\Delta\Delta Ct}$.

With two reference genes, it was possible to calculate minimum detectable difference in expression in an experiment from the ratios of reference gene pairings between control and treated or disease samples.

Due to the exponential amplification inherent to the process, it is important to ensure that the reference gene is not affected by the experimental conditions and that both the reference and genes of interest have similar amplification efficiencies [339].

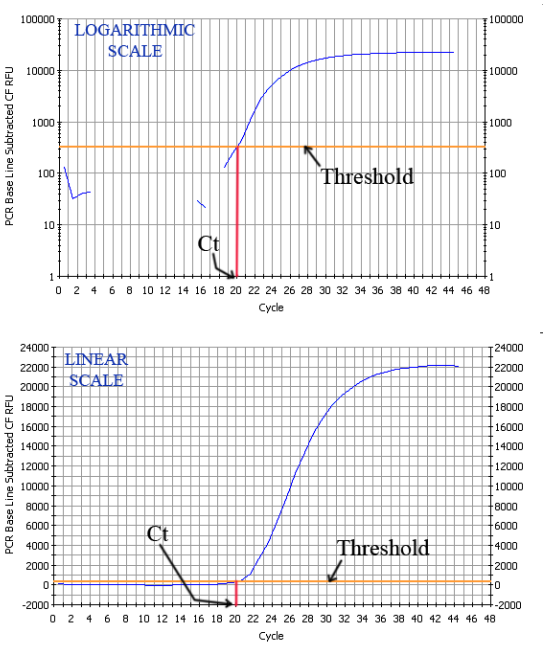

Figure 2.5. Real time PCR trace for one well on a logarithmic and a linear scale.

CHAPTER 3

Computed Tomography Airway Wall Thickness and its Relationship to Submucosal Collagen

3.1 The collagens

Collagens are the most abundant constituents of the extracellular matrix. They are a principal source of tensile strength and are responsible for the shape and physical properties of the tissues in which they occur.

3.1.1 General structure

Collagens are characteristically made up of 3 polypeptide chains arranged in a triple helix with long sequences of repeating Gly-Xaa-Yaa triplets. Around 28 different types of collagen are described, made up of 46 different polypeptide chains. The polypeptide α chain is arranged in a left-handed helix, with three chains then wrapping around each other to form a tightly-packed right-handed super-helix. The repeating Gly-Xaa-Yaa motif has Glycine, a small amino acid with no side-chain, which is able to fit within the inside of the triple-helix. The Xaa and Yaa can be any amino acid, but are most often proline or hydroxyproline. Hydroxyproline confers stability to the triple-helix at body temperature. Although some collagens are homotrimers made up of identical α chains, most are heterotrimers with different subunit combinations [340].

3.1.2 Classification

The fibrillar collagens (types 1, 2, 3, 5, 11, 24, 27) are the most studied and the most abundantly identified in vertebrate tissues. Other collagens have been described, which can be divided into different groups on the basis of their quaternary structure and functions. The network-forming collagens (types 4, 6, 8 and 10) form open networks rather than fibres and can provide anchorage for cells and probably also provide molecular filtration. Collagen types 9, 12, 14, 16, 19 – 22 and 26, collectively called FACITs (fibril-associated collagens with interrupted triple helices), are thought to act as molecular bridges which stabilise

the ECM. The membrane-associated collagens with interrupted triple helices (MACITs) include collagen types 13, 17, 23, and 25, have a trans-membrane domain thought to be important in cell-matrix interactions. Collagens 15 and 18 make up the MULTIPLEXINS (multiple triple helix domains and interruptions) and are closely associated with basement membranes [341].

The fibrillar collagens are the ones of most relevance vis-à-vis airway remodelling and will be discussed further.

3.1.3 Synthesis and regulation

The synthesis of the fibrillar collagens has been extensively studied.

The different α chain polypeptides are coded at different gene loci, but they all have a similar structural pattern. The triple-helical domain, coding for 1012 to 1029 amino acids is flanked by an N-terminal domain on one side and a C-terminal domain at the other. The C-propeptide domain (NC1) is highly conserved in fibrillar collagens, whilst the N-propeptide domain shows more variation among the different α chains. The N-terminal domain codes for a signal peptide and, depending on the specific polypeptide, a cysteine-rich repeat domain or a thrombospondin N-terminal-like domain with a variable region followed by a short triple-helical sequence. The C-propeptide is meant to be important in initiating and ensuring alignment of the triple-helix. The function of the N-propeptide is less clear.

Fibroblasts, and other cell types, synthesise protocollagen which is soluble and undergoes a number of post-translational modifications before forming collagen fibrils. These include cleavage of the signal peptide and hydroxylation of various amino acids. Hydroxylation is crucial to the stability of the molecule and prolyl-3- and prolyl-4-hydroxylase as well as various lysyl hydroxylases mediate this reaction at the different residue points. Some of these hydroxyl residues in turn

get galactose and glucose moieties added to them. The polypeptide is now referred to as procollagen and transits through the endoplasmic reticulum where trimerization occurs through the action of the C-propeptide. Three C-propeptides associate via disulfide bonds to form an initial contact point which then propagates towards the N-terminus to form the triple-helix [342].

Procollagen in parallel arrays is then transported towards the cell membrane in Golgi-to-plasma membrane carriers (GPCs). The next step is the cleavage of the non-helical propeptides through the action of procollagen-N-proteinase and procollagen-C-proteinase. It is still disputed whether this is occurring within the GPC or extracellularly within deep invaginations of the cell membrane [343]. The removal of the propeptides leads to the formation of insoluble tropocollagen consisting of a triple helix and short non-helical telopeptides at either end. The tropocollagens then self-assemble into collagen fibril intermediates. Through a series of lateral and end-to-end fusions, these intermediate fibrils enlarge into mature fibrils. The end telopeptides may be important to strengthen the formed fibrils through cross-links between lysine and hydroxylysine residues formed via the action of lysyl oxidase [341].

A number of molecules including FACIT collagens and small leucine-rich proteoglycans including decorin and biglycan, have emerged as important players in regulating fibrillogenesis [342].

3.1.4 Functions

The principal roles for the fibrillar collagens are provision of mechanical strength and ability to interact with other molecules.

Collagen 1 is a heterotrimer composed of two α1(I) chains and an α2(I) chain - $\{[\alpha1(I)]_2, \alpha2(I)\}$. It is ubiquitous in most tissues in the body except for cartilage and is the collagen most often associated with response to injury.

Collagen 3 is a homotrimer made of 3 identical polypeptides – $\{[\alpha 1(III)]_3\}$ and is found most abundantly in the walls of arteries and hollow organs [341].

3.1.5 Collagens in asthma

A number of studies have shown increased collagen expression in the bronchial wall in asthma. Although most focus has centred on subepithelial fibrosis which appears to consist mostly of collagens 1, 3 and 5 [65, 96, 127-129], increased submucosal collagen has also been shown [30]. This is not only restricted to the central airways but has also been shown to be present in peripheral airways [344].

An increase in this protein has been shown to be more pronounced in more severe disease [107, 195] and to inversely correlate with FEV_1 [141] and airway hyperresponsiveness [89]. A group of asthmatics subjected to allergen challenge had an upregulation of procollagen 1 and 3 seven days after exposure [345].

It is not clear what brings about the increased collagen. As the extracellular matrix is in a state of constant turnover, factors in both synthesis and degradation are likely to be relevant. Cytokines such as IL-4 have been shown to promote its production by human lung fibroblasts [346], providing a potential link with inflammation. Asthmatic fibroblasts have also revealed a dysregulation in matrix metalloproteinase production which could impair collagen breakdown [347].

Collagens are not mere structural bystander molecules but can modulate the behaviour of the airway cells. Collagen 1 can enhance airway smooth muscle proliferation and has been shown to confer resistance to inhibition of proliferation by steroids in vitro. It can also promote the secretion of pro-inflammatory cytokines by smooth muscle cells including eotaxin, GM-CSF and RANTES [348].

3.2 Aims

In asthma, there is increased airway wall thickness and increased collagen deposition. Both are manifestations of remodelling, but it is likely that a number of different processes contribute to remodelling. In order to evaluate the presence of a relationship between airway wall thickness and collagen deposition, immunohistochemical staining for submucosal collagens 1 and 3 in bronchial airway biopsies obtained from 3rd and 4th carinae and CT-derived airway dimensions from the same asthmatic and non-asthmatic subjects were compared.

The hypothesis thus being investigated is that thicker airways have increased collagen deposition.

3.3 Methods

3.3.1 Subject characteristics

A group of 14 healthy volunteers and 12 mild asthmatics was studied. The characteristics of the subjects are outlined in Table 3-1.

Table 3-1. Subject characteristics.

Disease	No.	Age* (yrs)	Sex (F:M)	FEV_1 pre-BD* (% predicted)	ICS* (mcg/day BDP equivalent)
Healthy	14	40.1 (28-54)	10 : 4	107 (84-125)	0
Asthma	12	37.2 (21-65)	5 : 7	90 (70-119)	467 (0-2000)

*Data shown as mean with range in brackets.
FEV_1 pre-BD = forced expiratory volume in 1 second as percentage predicted prior to bronchodilation.
ICS = inhaled corticosteroid, BDP = beclometasone dipropionate.

3.3.2 Computed tomography imaging

CT scans were performed on a GE systems HiSpeed CTi helical scanner (GE Systems, Milwaukee, WI, US) using a high resolution algorithm, collimation thickness 1mm, pitch 1.5mm, 120 KV and 180mA. Images from the level of origin of the right middle lobe to approximately 2cm above the right hemi diaphragm were captured while subjects were asked to take a full inspiration. This ensured cover of the basal segments of the right lower lobe. This yielded around 40 images from which 3 images showing the posterior segment of the right lower lobe bronchus perpendicularly were selected. For each subject, the thickness-to-diameter ratio (T/D) and percentage wall area (PWA) were measured using computer image software (Osiris Medical Imaging software v 4.19, University of Geneva, Switzerland) as shown in Figure 3.1.

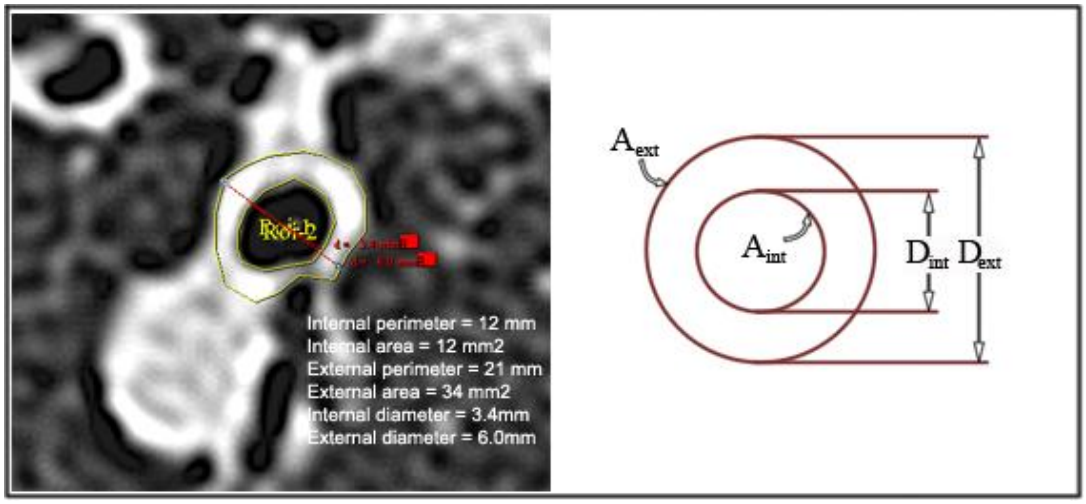

Figure 3.1. Computed tomography image of posterior segment of right lower bronchus (magnified x400) with schematic diagram of measurements taken.
T/D ratio calculated as $((D_{ext}-D_{int})/2)/D_{ext}$.
PWA calculated as $((A_{ext}-A_{int})/A_{ext}) \times 100$.

3.3.3 Endobronchial biopsy immunohistochemistry

Biopsies were obtained at bronchoscopy from segmental carinae as previously described and then stained for collagen 1 and collagen 3 as outlined in chapter 2. Mouse monoclonal antibodies against collagen 1 (1:500, Abcam, Cambridge, UK) and collagen 3 (1:1500, Chemicon, Temecula, US) were used on 2 micron sections of the GMA-embedded biopsies. Two non-contiguous sections from each sample were put on each slide for which collagen staining was subsequently assessed.

High resolution images of the biopsies were taken, and percentage area of staining quantified using computer-assisted image analysis. Using a Zeiss KS 400 image analysis system (Welyn Garden City, UK), a representative field was outlined in the submucosa, excluding blood vessels, damaged tissue and glands. Subepithelial collagen was excluded. The software would then calculate percentage area of staining within the delimited boundaries.

3.3.4 Statistics

Non-parametric statistical analysis was used to compare differences between the groups (Kruskal Wallis, Mann-Whitney U) and explore correlations (Spearman correlation coefficient). A p value of <0.05 was taken to denote statistical significance.

3.4 Results

3.4.1 Collagen immunostaining

Collagen 1 and 3 were present within the basement membrane and in the submucosa (Figure 3.2). The expression of both collagen 1 and collagen 3 as

determined by immunostaining was significantly increased in the asthma biopsies when compared to the non-asthmatic ones (Figure 3.3).

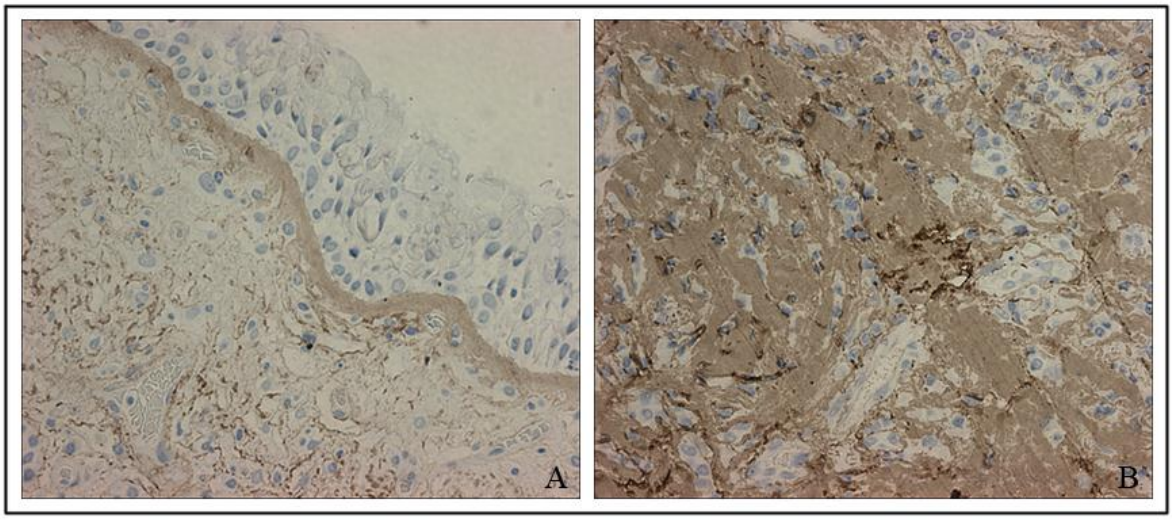

Figure 3.2. Representative images of collagen 1 (A) and collagen 3 (B) immunostaining.
Images shown at x40 magnification. Biopsies stained with isotype matched IgG control were completely clear.

3.4.2 Airway thickness by computed tomography

Both computed tomography measures of airway wall thickness – thickness-to-diameter ratio (Figure 3.4A) and percentage wall area (Figure 3.4B) – are significantly increased in the asthmatic subjects.

3.4.3 Correlations

No significant correlations were found between levels of immunostaining for either collagen 1 or collagen 3 and HRCT measures of airway wall thickness in asthmatics (Figure 3.5 and Figure 3.6).

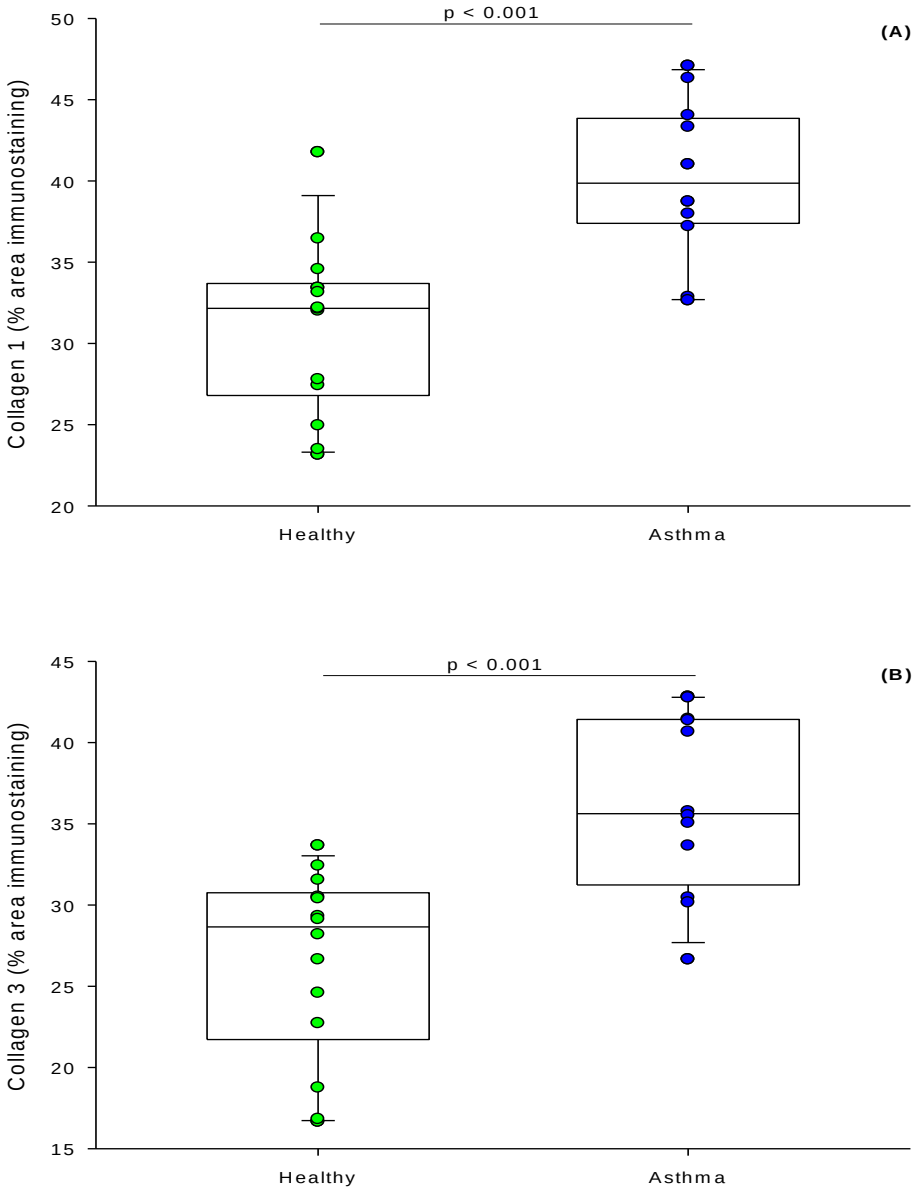

Figure 3.3. Submucosal collagen 1 (A) and collagen 3 (B) expression in endobronchial biopsies.
Data shown for non-asthmatic (n=14) and asthmatic (n=12) subjects.
Mann-Whitney U p <0.001 for both collagen 1 and collagen 3.

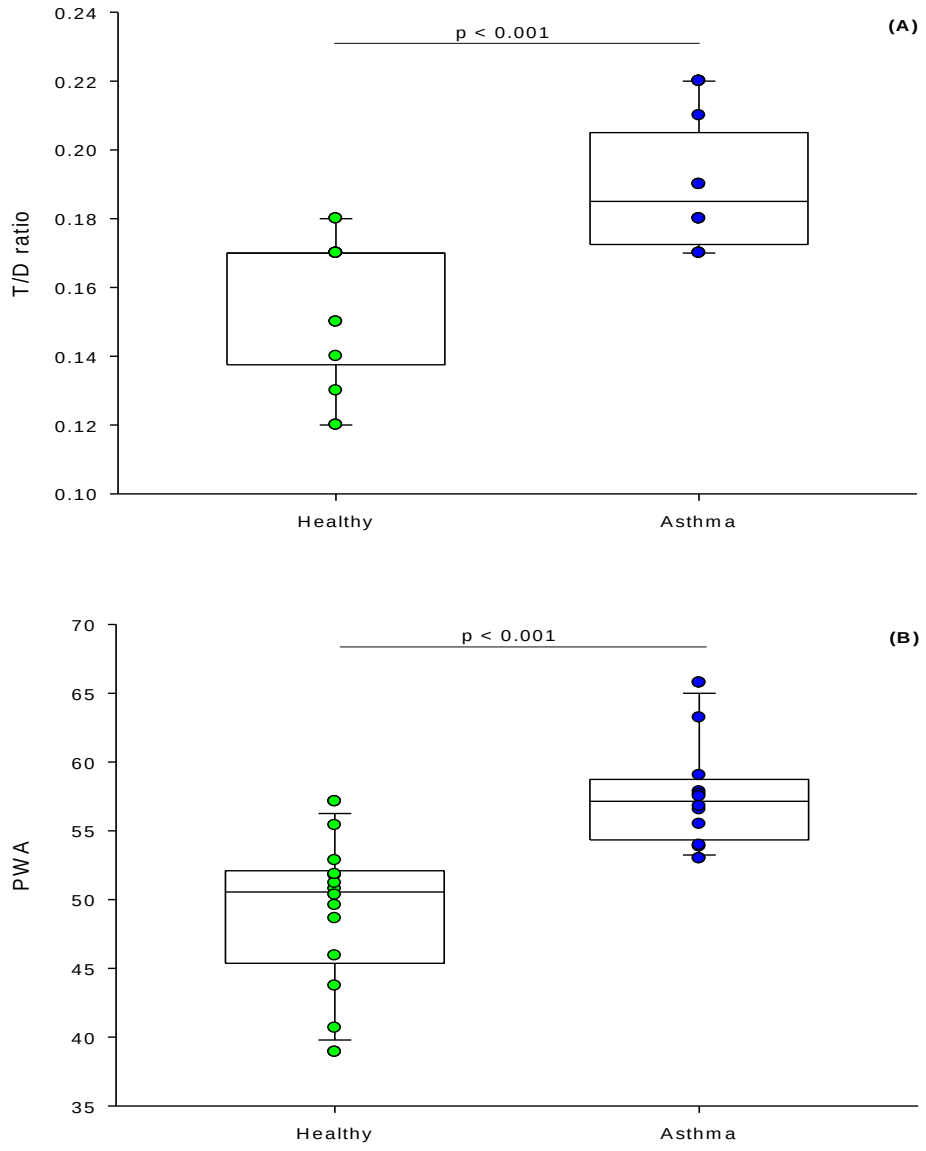

Figure 3.4. Thickness-to-diameter (T/D) ratio (A) and percentage wall area (PWA) (B) at the posterior segment of the right lower lobe bronchus as measured by high resolution CT scanning.
Data shown for non-asthmatic (n=14) and asthmatic subjects (n=12). Mann-Whitney U p <0.001 for both measurements.

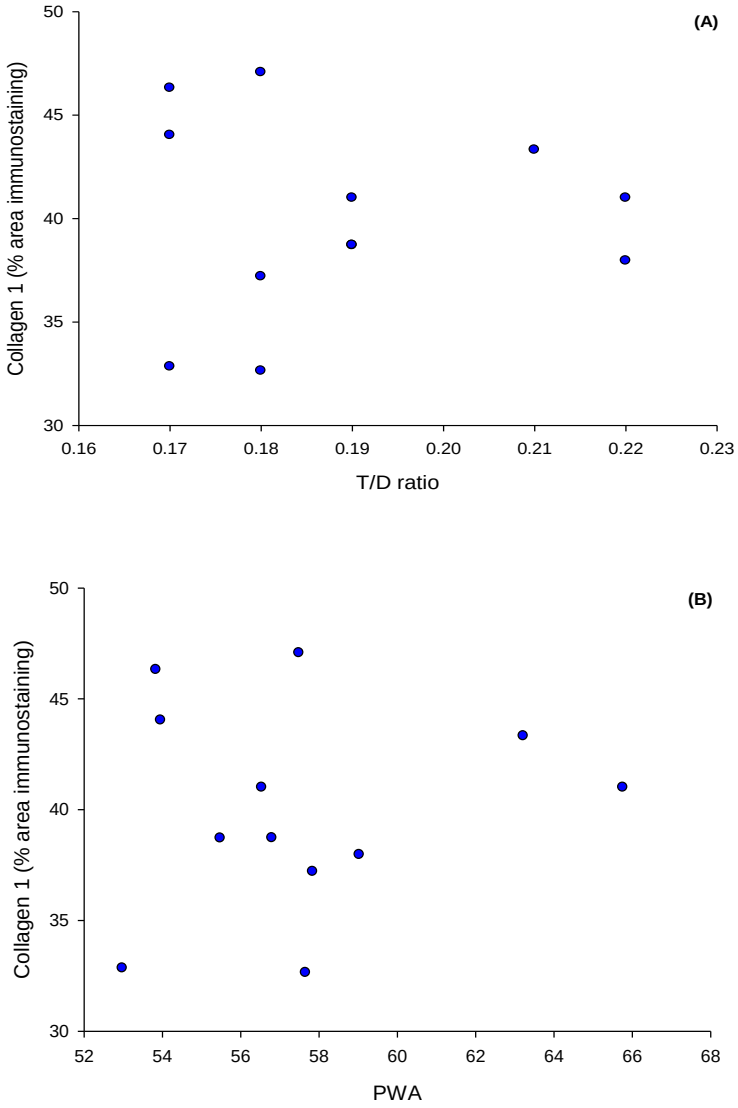

Figure 3.5. Correlation of collagen 1 percent area immunostaining with computed tomography measures of airway wall thickness.

(A) Thickness-to-diameter (T/D) ratio for asthmatic subjects (n=12) Spearman correlation coefficient r(12) = -0.077; p = 0.812.

(B) Percentage wall area (PWA) for asthmatic subjects (n=12) Spearman correlation coefficient r(12) = -0.081; p = 0.803.

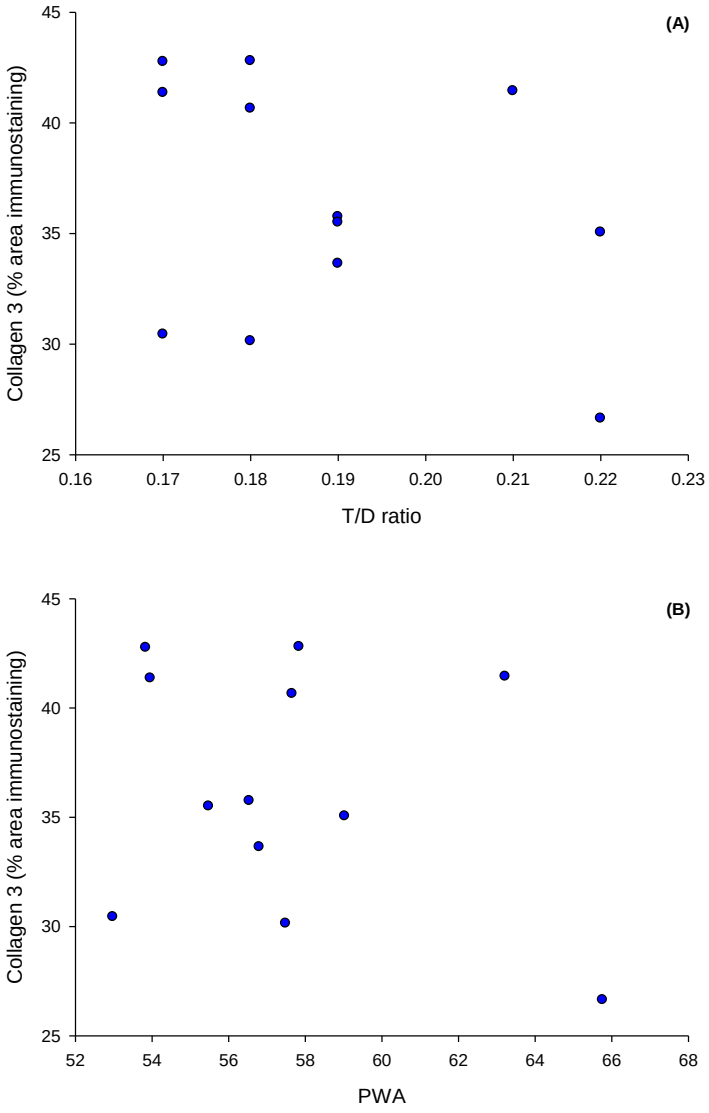

Figure 3.6. Correlation of collagen 3 percent area immunostaining with computed tomography measures of airway wall thickness.

(A) Thickness-to-diameter (T/D) ratio for asthmatic subjects (n=12) Spearman correlation coefficient r(12) = -0.329; p = 0.296.

(B) Percentage wall area (PWA) for asthmatic subjects (n=12) Spearman correlation coefficient r(12) = -0.098; p = 0.762.

3.5 Discussion

A number of factors contribute to the increase in the dimensions of the airway wall in asthma. As discussed previously, airway smooth muscle hyperplasia and hypertrophy, subepithelial fibrosis and epithelial changes as well as less easily measurable phenomena such as oedema all can potentially contribute to the thickening described. These data show a direct correlation between total wall thickness and collagen 1 and 3 presence. Correlation does not imply causality and it would be simplistic to infer that the increased wall thickness is caused by increased collagen deposition.

Collagen in the airway is produced principally, but not exclusively by fibroblasts and other mesenchymal cells such as smooth muscle cells. The increased collagen may reflect the upregulated synthetic capabilities of these cells or merely their increased presence within the airway wall.

The presence, concentration and level of activity of metalloproteinases in the airway wall would also be of relevance. Imbalances in the levels of metalloproteinases and their inhibitors have been shown in asthma [349, 350]. Matsumoto *et al* showed a negative correlation between measures of CT-derived airway wall thickness and sputum levels of matrix metalloproteinase-9 (MMP-9) and positive correlations with its inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1) in stable asthmatics [210]. This could be responsible for a decrease in the degradation and elimination of collagens.

Whatever the cause of the increased collagen, it is probably not merely having effects on the airway dimensions but also on the mechanical properties of the airway wall. Collagen has significant tensile strength and the increased presence of fibrils is probably contributing to the stiffness of the airway over and above the increase in volume. The alignment and orderly laying down of the collagen fibres has an important impact on the mechanical properties of the airways. In a study looking at a small number of asthmatics with mild asthma, airway distensibility, measured by a forced oscillation technique, was reduced relative to non-asthmatics. It was not related to lung elastic recoil, or bronchial muscle tone suggesting that this finding was a manifestation of airway remodelling [351].

Within the asthmatic group, the level of collagen expression does not correlate with airway wall thickness as measured by CT. Computed tomography measures whole wall thickness whilst collagen is presumably affecting specific layers within the wall. The group of asthmatics studied had a mild phenotype of the disease with no significant spirometric airflow impairment. Furthermore, the relatively low numbers do not provide sufficient power to confidently test the relationship within the asthmatics alone.

The extracellular matrix is an end-product of the fine-tuned balance of synthesis and degradation occurring within the bronchial wall. Any abnormalities in the expression of its constituents are suggestive of dysregulation which could be relevant to remodelling. In turn this could be significant in determining airway wall thickness and consequently clinical pathophysiology.

Airway wall thickness could therefore be associated with extracellular matrix dysregulation at a molecular level apart from physiological abnormalities.

CHAPTER 4

Endobronchial Ultrasound as a Tool in Assessing Bronchial Wall Layers and their Relationship to Airway Physiology in Asthma

4.1 Airway Remodelling

Airway remodelling refers to the measurable macroscopic and microscopic structural changes that occur in the bronchial wall of asthmatics. Changes have been described in all the components of the bronchial wall. There is evidence that the epithelium increases in volume [78], partly, but not entirely due to goblet cell hyperplasia which is accompanied by an increase in mucin production [77, 79]. One of the hallmarks of airway remodelling is increased deposition of collagen in the lamina reticularis resulting in the typical histopathological finding of a thickened reticular basement membrane [88, 128, 352, 353]. The smooth muscle mass also increases as a consequence of smooth muscle cell hypertrophy and hyperplasia [107, 354]. Other features of remodelling include, oedema and an increase in the number of blood vessels [355].

There is a paucity of studies that actually correlate whole airway wall thickness with any of the histopathological changes outlined above. Kasahara *et al* showed a correlation between reticular basement membrane thickness and airway wall dimensions in 49 subjects with mild and moderate asthma [207]. However it seems reasonable to assume that the pathological changes described above result in an overall thickening of the airway wall.

4.2 Airway Wall Thickening

Airway wall thickening is probably the most obvious manifestation of remodelling. A number of studies have, over the years, investigated the physical dimensions of the airway wall in asthma using various means. Although making comparisons between studies is not always easy, the increased thickness of the airway is an established fact.

4.2.1 Post-mortem studies

Initial anecdotal reports of airway wall thickening in fatal asthma post-mortems, prompted investigators to embark on formal studies measuring the airway wall parameters. Many of these studies compare cases of fatal asthma with non-asthma deaths, which limits extrapolation of the findings to the general asthmatic population.

James *et al* showed an increased total wall area arising from larger epithelial, submucosal and smooth muscle areas in 18 cases of asthma (11 of which were fatal asthma) compared to 23 non-asthmatic controls [105].

Further studies showed a graded increase in thickness of post-mortem airways from non-asthmatics to nonfatal asthma and then to fatal asthma. This was shown in the adventitial, smooth muscle and submucosal compartments of the membranous airways [356]. Others demonstrated that the increased area in asthma occurred at all levels of the bronchial tree [78].

4.2.2 High-resolution computed tomography studies

With the advent of high-resolution computed tomography (HRCT), an imaging modality that lent itself to the measurement of airway dimensions became available [357, 358]. A number of studies, summarised in Table 4-1, have documented the airway thickening referred to in post-mortem studies, extending the findings to living subjects and including less severe forms of asthma in their analyses [209]. There is a lot of heterogeneity in these studies in terms of the population sampled, the technology used and the way the measurements are taken.

A number of these studies have demonstrated increased airway thickening in asthma [202, 203, 207, 209], which also seems to correlate with the severity of

the disease [204-206]. Equating the airway wall dimensions with physical parameters of asthma has proved more difficult. An inverse relationship with FEV_1 has been shown in some studies [205, 207, 209] but not in others [203, 206]. One study demonstrated that the longer the duration of asthma the thicker the airways [205]. Airway wall thickness has been correlated with airway hyperresponsiveness in some [201] but not in all studies [203]. Indeed, Niimi *et al* described a negative correlation between airway wall thickness and airway hyperreactivity [143].

4.2.3 Endobronchial ultrasound

Ultrasound in airway imaging has been mostly applied in the assessment of bronchial wall tumour invasion and for guiding needle biopsies of peribronchial lymph nodes [359]. Five distinct echo bands are described in ultrasonographic imaging of the bronchial wall – alternating hyper- and hypo-echoic bands generated by the interphase between tissues of different densities. These bands have been correlated with the components of the bronchial wall [328, 360, 361]. Kurimoto *et al*, using resected human trachea and bronchi, inserted needles under ultrasonographic guidance into the different ultrasound layers. These specimens were then examined histologically and the puncture hole site correlated with the ultrasound image [328].

The first hyperechoic band is formed at the boundary of the lumen and epithelium. The hyperechoic area extends into the submucosa, gradually fading into the first hypoechoic band. The next boundary occurs at the inner border of the airway cartilage. The hypoechoic region between the first and second hyperechoic bands therefore corresponds to submucosa and smooth muscle layers. The second hyperechoic band merges into the second hypoechoic band, both together representing the cartilage. At the boundary between cartilage and adventitia, a third hyperechoic region is generated (Figure 4.1).

Table 4-1. Summary of select HRCT studies in asthma.

Study	Subjects	Site measured	Measures used	Results			
				Group comparisons	Correlations		
					Positive	Negative	Absent
Boulet *et al* 1995 [201]	14 asthma irreversible obstruction 11 asthma reversible obstruction 10 healthy controls	Right bronchus intermedius	T/D	–		PC20 in irreversible asthma	FEV1%
Okazawa *et al* 1996 [202]	6 mild asthma 6 healthy controls	Various bronchi 2 levels	WA	–			
			WA%	↑ (asthma)			
Awadh et al 1998 [204]	14 near fatal asthma 12 moderate asthma 13 mild asthma 14 healthy controls	Bronchi luminal diam >1mm 5 levels	T/D	↑ (near fatal + moderate asthma vs mild asthma) ↑ (all asthma groups vs healthy)			
			WA%				
Niimi *et al* 2000 [205]	22 severe asthma 39 moderate asthma 13 mild asthma 7 intermittent asthma 28 healthy controls	Apical bronchus right upper lobe	WA	↑ (mild, moderate + severe asthma groups vs healthy controls)	Asthma duration and severity	FEV1% FEV1/FVC%	
			WA/BSA				
			WA%			FEV1%	
Little *et al* 2002 [206]	49 mild/moderate asthma	Bronchi luminal diam >1.5mm 5 levels	T/D		Asthma severity ATS score	KCO%	FEV1% Induced sputum cells FeNO
			WA%				
Kasahara *et al* 2002 [207]	49 mild/moderate asthma 18 healthy controls	Bronchi luminal diam 3-5mm 5 levels	WA%	↑ (asthma)	RBM	FEV1%	
			WT%				
Gono *et al* 2003 [209]	24 asthma irreversible obstruction 10 asthma reversible obstruction 7 healthy controls	Apical bronchus right upper lobe	T/D	↑ (irreversible asthma vs other groups)		FEV1%	
			WA%				

ATS – American Thoracic Society, BSA – body surface area, diam – diameter, FeNO – fraction of exhaled nitric oxide, FEV1% – forced expiratory volume in 1s percent of predicted, FVC% – forced vital capacity percent of predicted, KCO% – carbon monoxide transfer coefficient percent of predicted, RBM – reticular basement membrane, T/D – thickness-to-diameter ratio, WA – wall area, WA% – wall area to total area percent, WT% – wall thickness to whole thickness percent

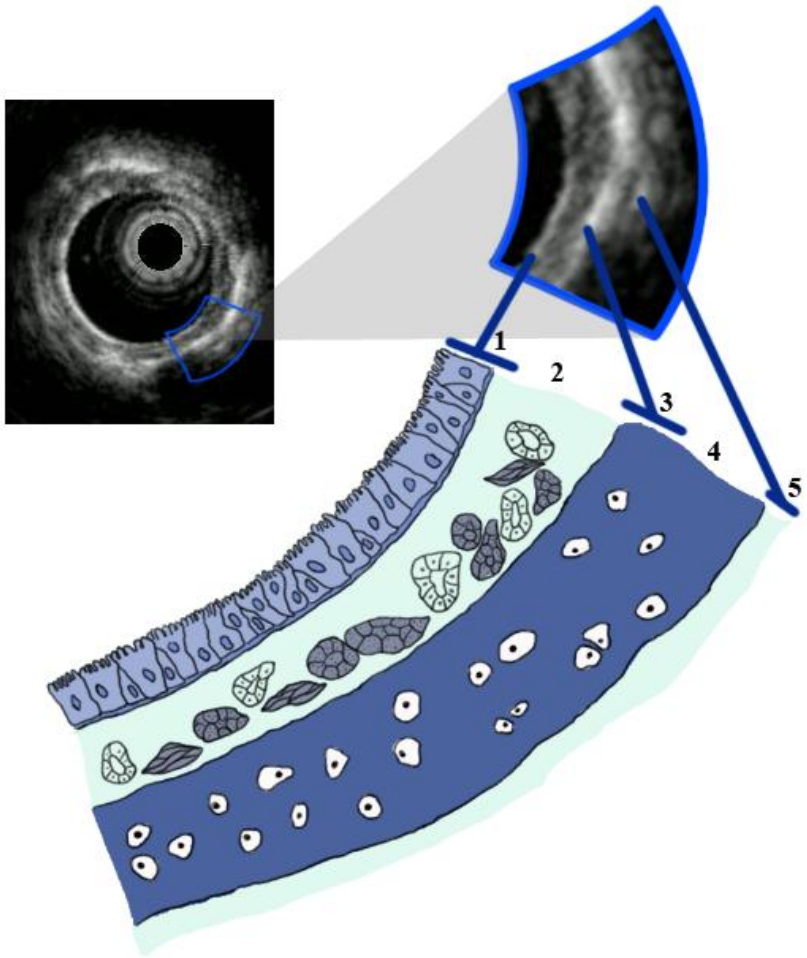

Figure 4.1. Echo bands on bronchial wall ultrasonography with corresponding tissue layers.
First hyperechoic band (1) corresponds to epithelium and part of submucosa. First hypoechoic band (2) corresponds to submucosa. Second hyperechoic (3) and second hypoechoic (4) bands correspond to bronchial wall cartilage. Third hyperechoic band (5) corresponds to adventitia.

4.3 Aims

Increased airway wall thickness has been shown in asthma but it is less clear how this relates to airway mechanical physiology. More severe asthmatics, particularly those with a fixed airflow defect, would presumably have thicker airway walls. Using endobronchial ultrasonography it may be possible to distinguish separate components of the wall and identify differential thickening.

This work hypothesises that severe asthmatics, particularly those with a fixed airflow defect have thicker airway walls as measured by endobronchial ultrasound.

4.4 Methods

4.4.1 Endobronchial ultrasound

Bronchoscopy with ultrasound was performed as described in Chapter 2 section 2.3 and 2.4. Briefly, endobronchial ultrasound was performed at the bronchoscopy by introduction of a radial ultrasound probe enclosed in an inflatable water-filled balloon sheath through the working channel of the bronchoscope. This was passed into the distal right lower lobe bronchus. The balloon was inflated just outside the orifice of the posterior basal segmental bronchus to achieve sonic coupling and allow images to be obtained and recorded.

4.4.2 Choosing the still image

Video images of the inflation / deflation were stored in the hard disc of the ultrasound machine and analysed retrospectively in a blinded fashion. Still images were chosen from the video, stored as TIFF (tagged image file format) images and transferred to a personal computer for further analysis. In order to try to eliminate the potential airway wall distortion that the inflation of the balloon could introduce, the point when a good image of the airway wall was obtained at least inflation was chosen. In effect, since the video usually included both balloon inflation and deflation, often more than one image could be obtained. Between 1 and 8 images (mean 2.6 images) were used per subject to derive the measurements.

4.4.3 Measurements

Three ratios reflecting airway wall thickness were derived from the images – two ratios representing whole wall thickness and one ratio looking at partial wall thickness. The percentage wall area (PWA) and thickness-to-diameter ratio (T/D) are commonly quoted measures in HRCT studies. The percentage inner wall area (PIWA) measures the inner layers of the airway wall.

Measurements were performed using computer software – Osiris (Osiris Medical Imaging software v 4.19, University of Geneva, Switzerland). After calibration this allowed measurement of lengths and areas on the chosen still images.

The measurements were made in a blinded fashion and then used to derive ratios as shown in Figure 4.2, Figure 4.3 and Figure 4.4.

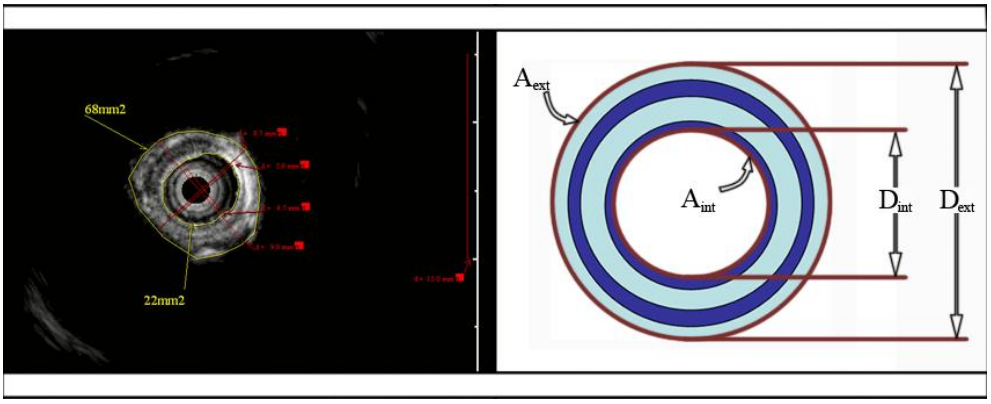

Figure 4.2. Areas and lengths measured for deriving Percentage wall area and Thickness-to-diameter ratio.
A_{ext} = total wall area, A_{int} = luminal area, D_{ext} = external diameter, D_{int} = internal diameter.

4.4.3.1 Percentage wall area

The luminal border of the airway was outlined, giving a measure termed the internal area (A_{int}). This is usually a sharply defined border as it lies at the boundary between the water of the balloon sheath and the epithelial layer, which have very different acoustic impedances.

The outer border of the airway is slightly more problematic. The outer limit of the second hypoechoic band was used to delineate the total wall area. This corresponds to an area including the cartilage plates of the airway. Due to loss of energy with deeper penetration of the ultrasonic waves as well as the absence of a sharp acoustic border, the resulting image tends to be rather grainy and ill-defined at this level. In order to maintain consistency, the border was drawn along points of intermediate echogenicity between the most intense and least intense signal. From this outline, the external area (A_{ext}) was measured (Figure 4.2).

The percentage wall area (PWA) is then calculated from the percentage ratio of the difference between the external and internal areas to the external area.

$$\mathrm{PWA}=\frac{\mathrm{A_{ext}}-\mathrm{A_{int}}}{\mathrm{A_{ext}}}\times 100$$

4.4.3.2 Thickness-to-Diameter ratio

Diameters were measured at the shortest and longest axis of the airway. Two diameters were measured at these axes. The external diameter ran across the external area whilst the internal diameter joined the corresponding edges of the luminal or internal area (Figure 4.2).

The thickness-to-diameter ratio (T/D ratio) is derived by subtracting the internal diameter from the external diameter and dividing by two and taking the ratio of the result to the external diameter.

$$\mathrm{T/D\ ratio}=\frac{(\mathrm{D_{ext}}-\mathrm{D_{int}})/2}{\mathrm{D_{ext}}}$$

The above two indices are the ones most commonly used in measuring airway wall dimensions in HRCT.

4.4.3.3 Inner wall area

Due to the ill-defined outer border of the airway on ultrasound, other methods of measurement were also adopted. The area between the luminal border and the inner perimeter of the second hyperechoic band is usually better defined in these images and would, in theory, correspond to the combined areas of the epithelium, submucosa and smooth muscle layers. This is here referred to as the inner wall area (IWA).

Two methods of measuring this area were used.

The first method (Figure 4.3) was an attempt to address the fact that very often a sector of the whole circumference of the airway is imaged with better definition than the rest. To circumvent this problem, radii were drawn from the centre of the lumen to the edges of the region of well-defined airway wall. The angle (θ) subtended by this sector was recorded. The combined area of the first two echo bands (first hyperechoic and first hypoechoic regions – A_{sec}) was measured and multiplied, using the angle θ, to extend that area around a full 360°.

$$\mathrm{IWA} = \frac{360^\circ}{\theta} \times \mathrm{A_{sec}}$$

This method obviously makes a number of assumptions. The determination of the centre of the airway, which is never a perfect circle, is based on a visual impression rather than a geometric calculation. By extrapolating the measured area, it is being presumed that the airway is a perfect circle and that the measured area is remaining constant all around.

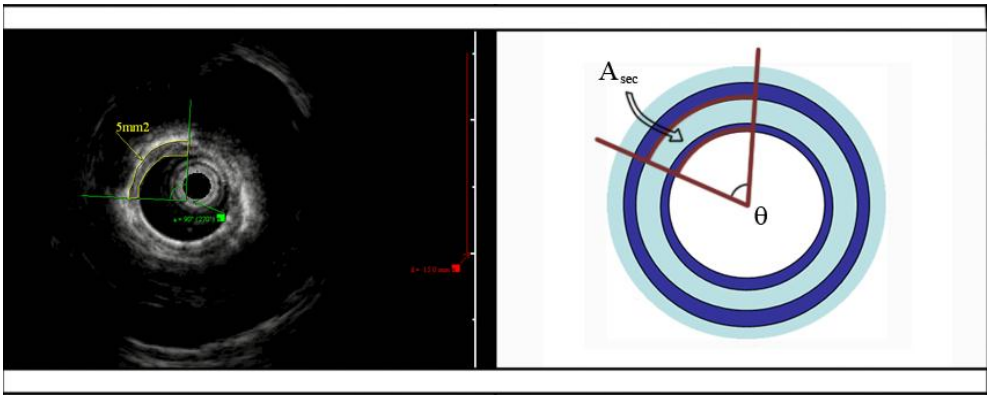

Figure 4.3. Areas and lengths measured for deriving inner wall area by sector extrapolation method.
A_{sec} = area of sector measured directly, θ = angle subtended by sector at centre.

In the second method (Figure 4.4), direct measurements were made of the luminal area (A_{int}) and the total inner area (A_{inner}). The inner wall area was then derived by subtraction of the two.

$$\mathrm{IWA} = \mathrm{A_{inner}} - \mathrm{A_{int}}$$

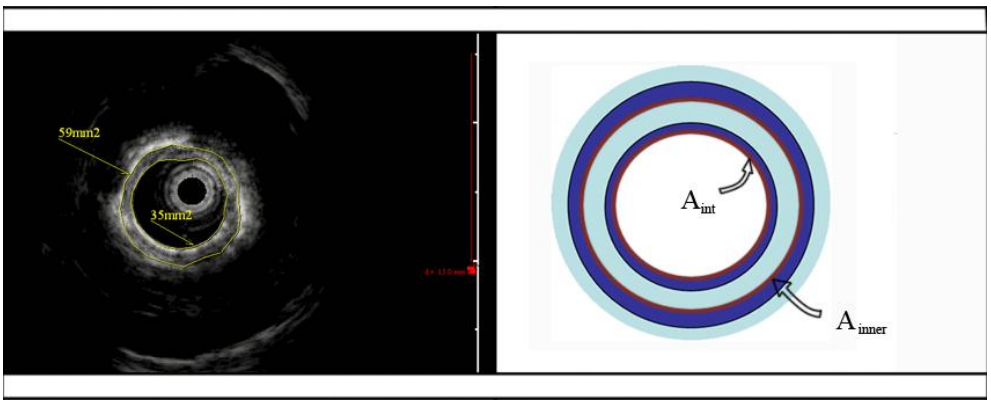

Figure 4.4. Areas measured for deriving inner wall area by direct measurement.
A_{int} = luminal area, A_{inner} = inner area including luminal area and first two echo bands.

Both measures were normalised by deriving a ratio to the total inner area – giving a percentage inner wall area (PIWA).

$$\mathrm{PIWA} = \frac{\mathrm{IWA}}{\mathrm{A_{inner}}} \times 100$$

4.4.4 Statistics

Non-parametric statistical analysis (Kruskal Wallis, Mann-Whitney U) was used to compare differences between the groups. Pearson and Spearman correlation coefficients were used to explore correlations. A p value of <0.05 was taken to denote statistical significance.

4.5 Subject characteristics

Endobronchial ultrasound data were obtained from a total of 38 subjects – 15 healthy controls and 23 asthmatics. Out of the 51 subjects recruited, 6 failed to have endobronchial ultrasound for various reasons, and 7 ultrasound videos were deemed to be of too low a quality to allow reliable measurements to be made.

Two classification methods were adopted.

- In the first instance, asthmatics were classified using the Global Initiative for Asthma (GINA) classification [1]. On the basis of this, the 23 asthmatics were separated into 12 subjects with severe persistent asthma (referred to as severe asthma) and 11 subjects with mild / moderate asthma (4 with intermittent asthma, 3 mild persistent asthmatics, and 4 moderate persistent asthmatics). The characteristics of the subjects are outlined in the Table 4-2.

Table 4-2. Subject characteristics when categorised by GINA classification.

Disease	No.	Age* (yrs)	Sex (F :M)	FEV_1 pre-BD* (% predicted)	ICS* (mcg/day BDP equivalent)	PC_{20} # (number + geomean)
Healthy	15	35.1 (19-64)	6 : 9	102.9 (79.2-133.8)	0	15 >16mg/ml
Mild / moderate asthma	4 I 3 MP 4 MoP	32.8 (18-64)	8 : 3	93.3 (68.7-113.4)	223 (0-1000) 4 I + 1 MP not on ICS	9 5.6mg/ml
Severe asthma	12 SP	46.1 (17-71)	9 : 3	71.5 (35.6-125.3)	1698 (800-2800)	4 3.8mg/ml

*Data shown as mean with range in brackets.
#Number of subjects who had a bronchial challenge and PC_{20} geomean are shown.
Bronchial challenge not performed in 2 moderate asthmatics and 8 severe asthmatics as not deemed clinically appropriate.
SP = severe persistent, MoP = moderate persistent, MP = mild persistent and I = intermittent asthma.
FEV_1 pre-BD = forced expiratory volume in 1 second as percentage predicted prior to bronchodilation.
ICS = inhaled corticosteroid, BDP = beclometasone dipropionate.
PC_{20} = methacholine concentration causing a 20% drop in FEV_1.

- The second method of classification is based on the airway obstructive defect as determined by the percent predicted FEV_1 and its reversibility. All non-asthmatics were considered as having "normal" airways. The asthmatics were divided in two groups. The first group with a "reversible" airway obstruction either started off with a percent predicted FEV_1 above 80% or achieved this after having nebulised salbutamol. The second group who never achieved a percent predicted FEV_1 of above 80% were deemed to have a "fixed" airway obstruction. The subject characteristics when grouped in this way are summarised in Table 4-3.

Table 4-3. Subject characteristics when categorised by airflow defect.

Airflow defect	No.	Age* (yrs)	Sex (M : F)	FEV_1 pre-BD* (% predicted)	ICS* (mcg/day BDP equivalent)	PC_{20} # (number + geomean)
Normal	15	35.1 (19-64)	9 : 6	102.9 (79.2-133.8)	0	15 >16mg/ml
Reversible	13	32.2 (18-64)	3 : 10	96 (69.3-125.3)	527 (0-2400) 5 not on ICS	10 4.4mg/ml
Fixed	10	50 (17-71)	3 : 7	62.8 (35.6-77.8)	1587 (0-2800)	3 7.9mg/ml

*Data shown as mean with range in brackets.
#Number of subjects who had a bronchial challenge and PC_{20} geomean are shown.
Bronchial challenge not performed in 2 moderate asthmatics and 8 severe asthmatics as not deemed clinically appropriate.
FEV_1 pre-BD = forced expiratory volume in 1 second as percentage predicted prior to bronchodilation.
ICS = inhaled corticosteroid, BDP = beclometasone dipropionate.
PC_{20} = methacholine concentration causing a 20% drop in FEV_1.

4.6 Results

4.6.1 Percentage wall area

Analysis by percentage wall area failed to reveal significant differences between the groups. Figure 4.5A and Figure 4.5B show the values for PWA divided by asthma severity and airways disease respectively.

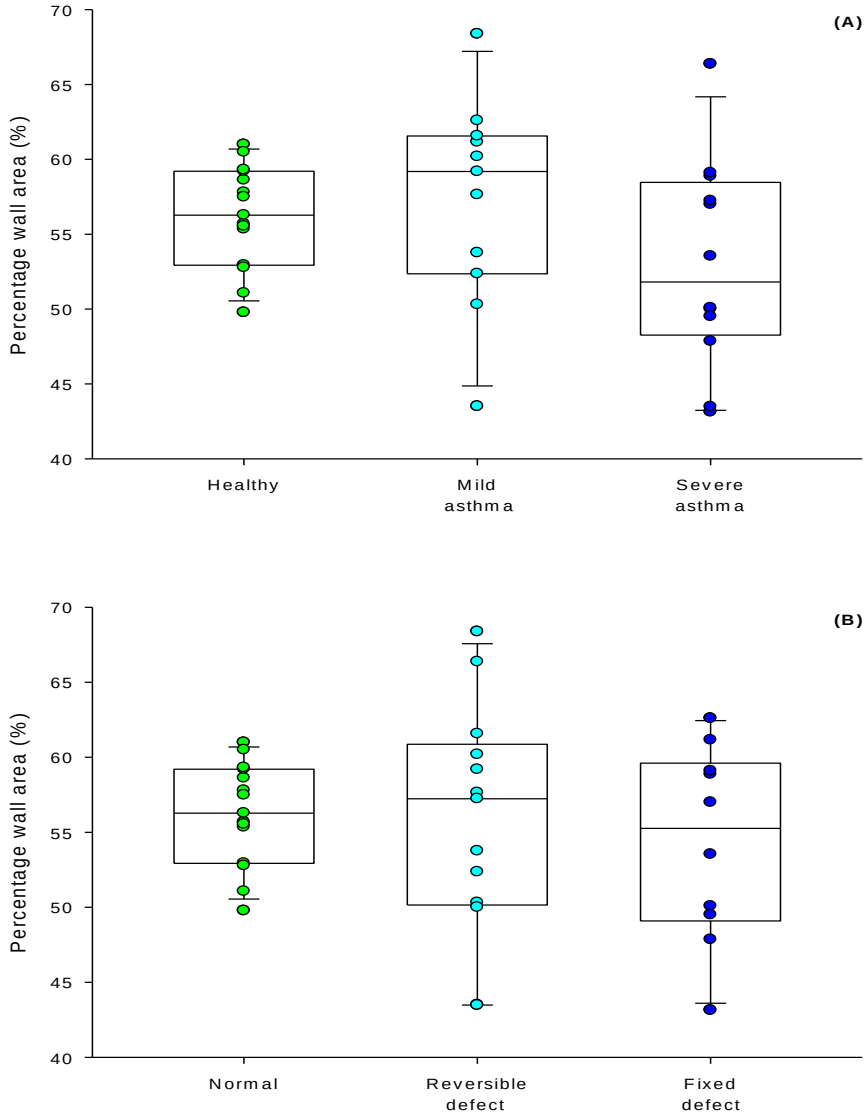

Figure 4.5. Percentage wall area by disease severity (A) and airflow defect (B).
(A) Subjects were divided into non-asthmatics (n=15), mild asthmatics (n=11) and severe asthmatics (n=12). Kruskal Wallis p = 0.109.
(B) Subjects were divided into those with normal airflow (n=15), reversible (n=13) and fixed (n=10) airflow defect. Kruskal Wallis p = 0.799.

4.6.2 Thickness-to-Diameter ratio

Diameters were measured at the longest and the shortest axis of the ultrasound image of the airway. It proved difficult to determine which one of the two best represented the wall thickness. On one hand the shorter diameter may be reflecting unequal wall compression by the inflated balloon sheath at various points around the lumen. On the other hand, if the plane of the ultrasound waves is not perpendicular to the true cross-section of the wall a thicker wall will be imaged as the waves are crossing it transversely (Figure 4.6). Both diameters were measured, and when compared they appear to correlate very closely (Figure 4.7).

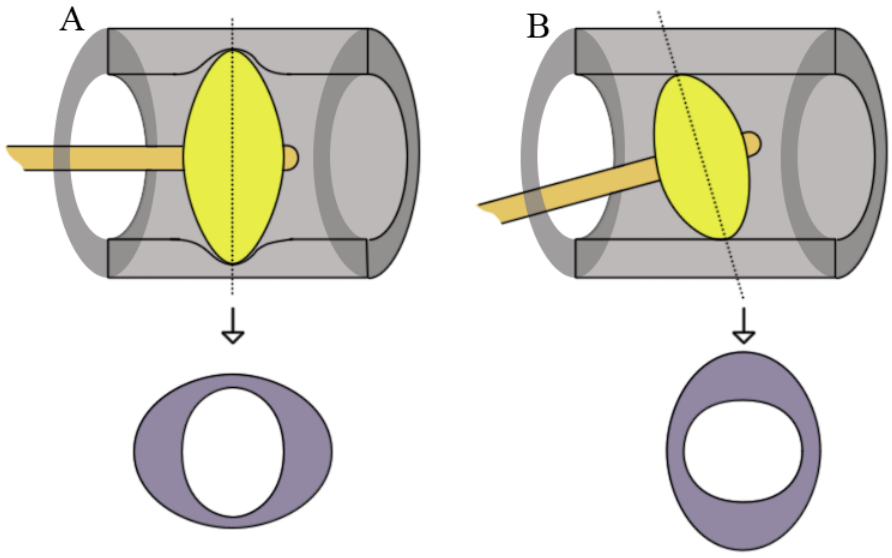

Figure 4.6. Potential reasons for different measures of diameter on endobronchial ultrasound.
(A) Unequal compression of airway wall by balloon.
(B) Non-parallel probe.

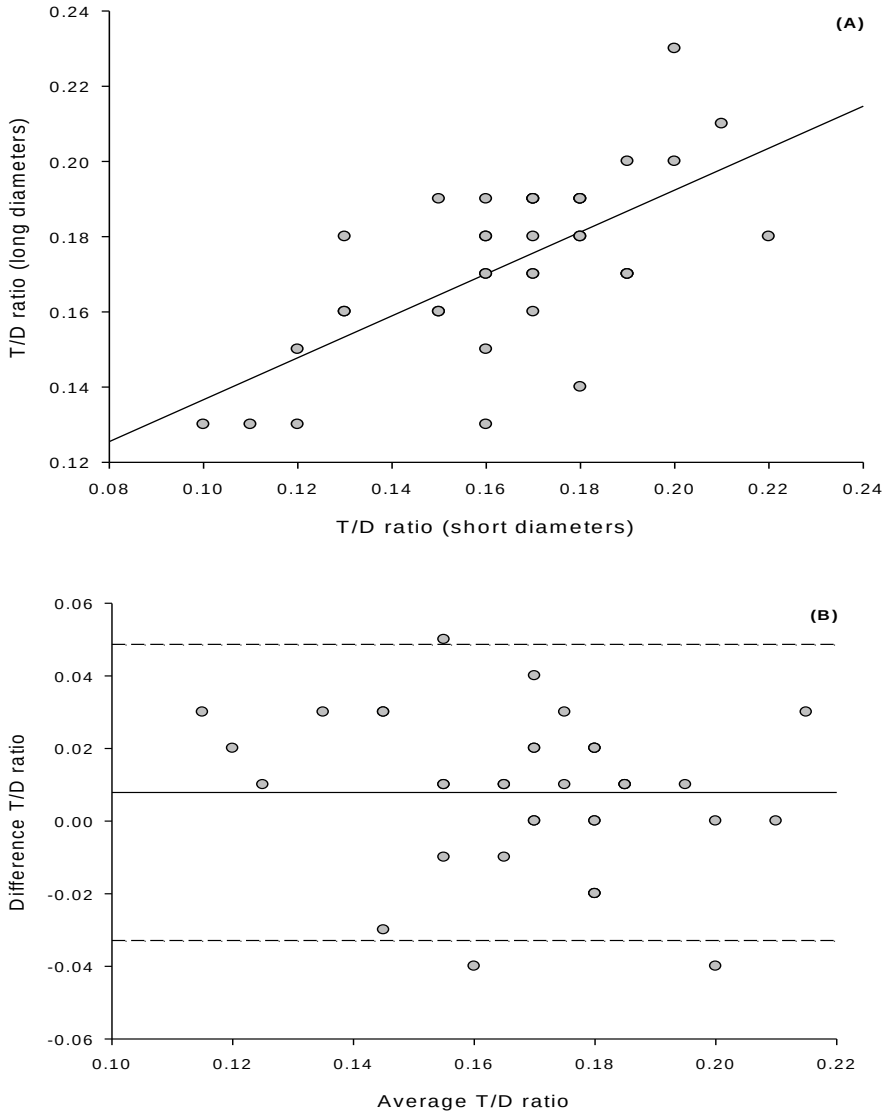

Figure 4.7. Comparison of measurements of T/D ratios using short and long diameters of airway image showing correlation (A) and Bland-Altman plots (B).
(A) Pearson correlation coefficient $r(38) = 0.666$; $p < 0.001$. $r^2 = 0.443$.
(B) Mean(difference) is shown by solid line. Mean(difference) ±1.96SD is shown by broken lines.

The short ratio was used for all subsequent analyses as there is potentially more likelihood of eliminating compression artefacts by choosing the right image.

There was no statistically significant difference in T/D ratio between non-asthmatic, mild asthmatic and severe asthmatic airway walls (Kruskal Wallis $p = 0.152$) as shown in Figure 4.8A.

The analysis was also done with the subjects divided according to the nature of the airway abnormality (Figure 4.8B). There was no statistical difference in the averages of the groups (Kruskal Wallis $p = 0.765$).

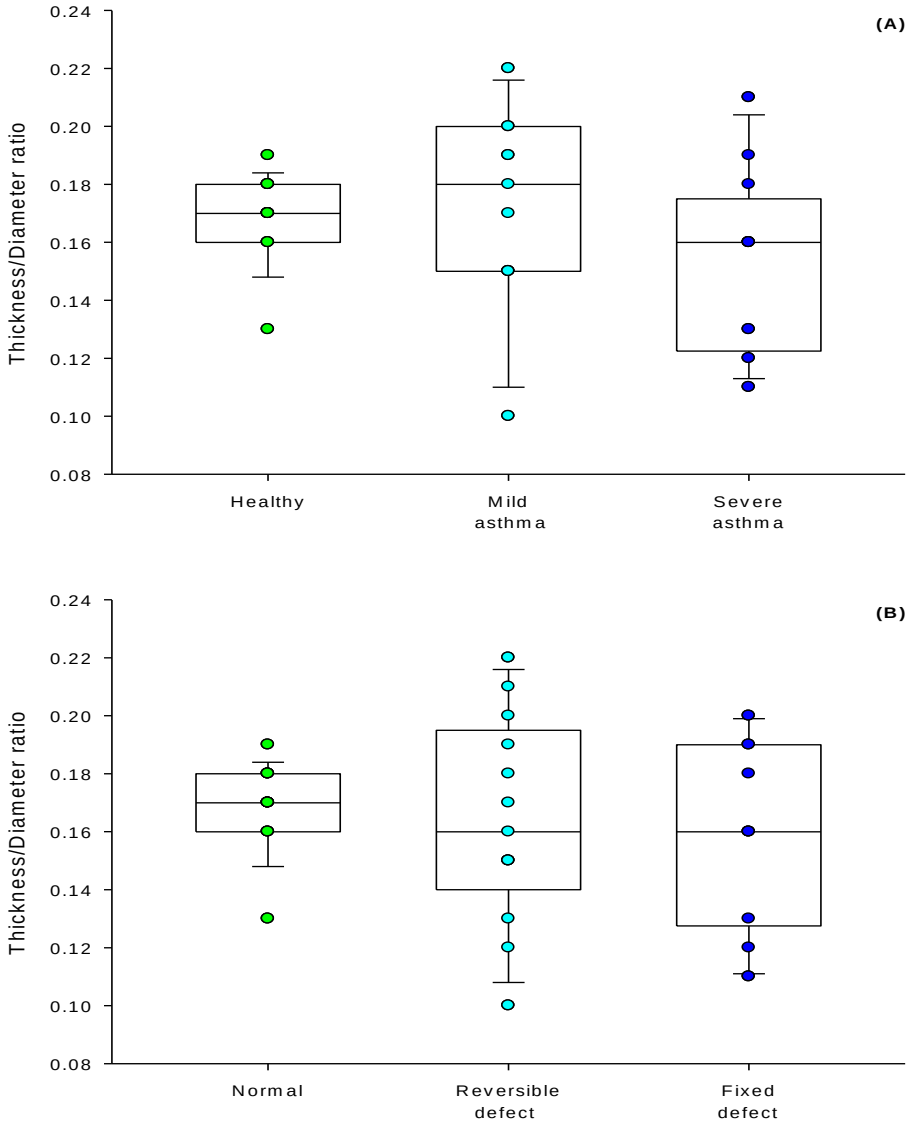

Figure 4.8. Thickness-to-diameter ratio by disease severity (A) and airflow defect (B).

(A) Subjects were divided into non-asthmatics (n=15), mild asthmatics (n=11) and severe asthmatics (n=12).

(B) Subjects were divided into those with normal airways (n=15), reversible (n=13) and fixed (n=10) airflow defect.

4.6.3 Inner wall area

Inner wall area consisting of the first two echo bands, representing the epithelial and submucosal components of the airway wall, was measured in two different ways – by direct measurement and by extrapolation of the measurement of the best defined sector as described earlier. The two measures were highly correlated, and consequently the direct measure, which is less dependent on extrapolations, was used in the subsequent calculations (Figure 4.9).

In order to correct for minor variations in the site of inflation of the balloon sheath, the inner wall area was divided by the total inner area (A_{inner}) giving the percentage inner wall area (PIWA). When these parameters are plotted, no difference can be seen between the groups studied (Figure 4.10).

The denominator of the calculated PIWA, A_{inner} is the sum of the luminal area and the inner wall area. Plotting the luminal area by disease severity and airflow defect (Figure 4.11), shows that this measure is changing significantly with disease status and airflow limitation.

In fact when taking into consideration the non-normalised inner wall area, as seen in Figure 4.12A and Figure 4.12B, there are statistically significant differences when the asthmatics are categorised by severity (Kruskal Wallis $p = 0.049$) and more so when they are divided by airflow defect (Kruskal Wallis $p = 0.006$).

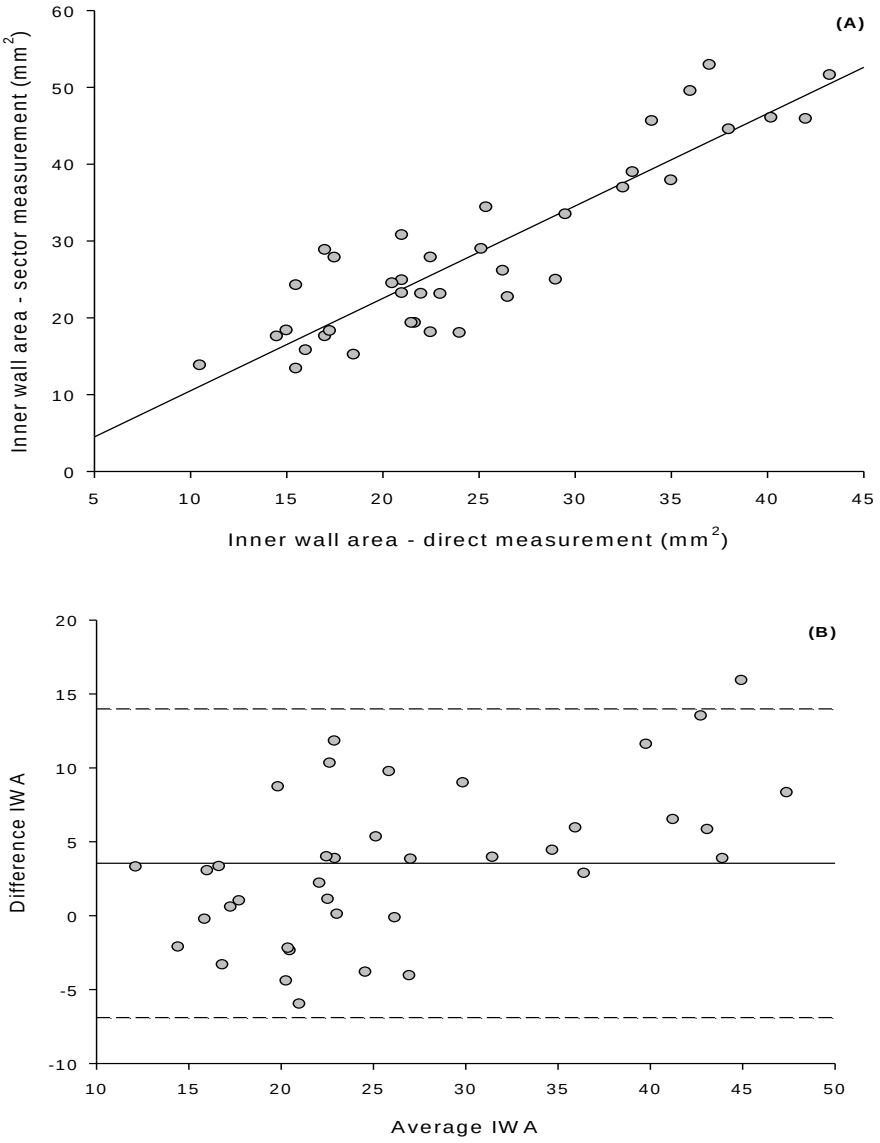

Figure 4.9. Comparison of measurements of inner wall areas using sector and direct methods showing correlation (A) and Bland–Altman plots (B).
(A) Pearson correlation coefficient $r(38) = 0.898$; $p < 0.001$. $r^2 = 0.807$.
(B) Mean(difference) is shown by solid line. Mean(difference) ±1.96SD is shown by broken lines.

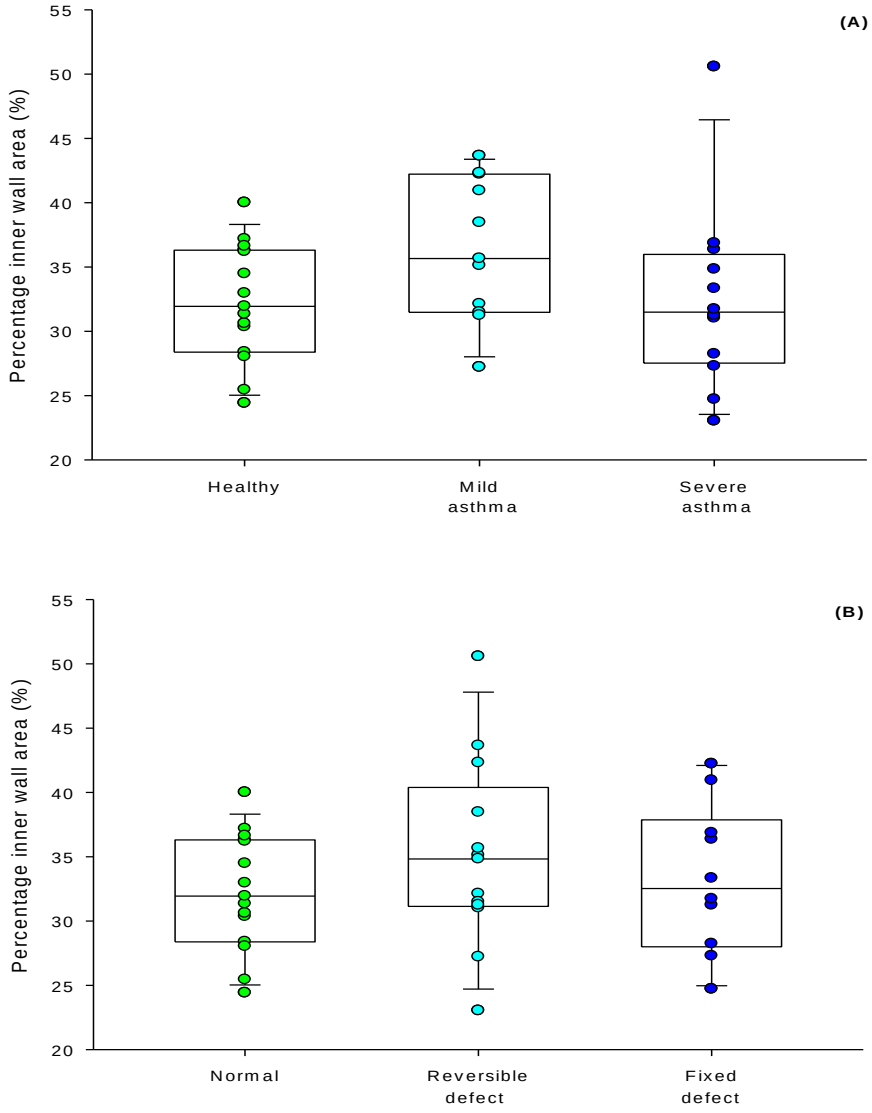

Figure 4.10. Percentage inner wall area by disease severity (A) and airflow defect (B).

(A) Subjects were divided into non-asthmatics (n=15), mild asthmatics (n=11) and severe asthmatics (n=12). Kruskal Wallis p = 0.134.

(B) Subjects were divided into those with normal airflow (n=15), reversible (n=13) and fixed (n=10) airflow defect. Kruskal Wallis p = 0.629.

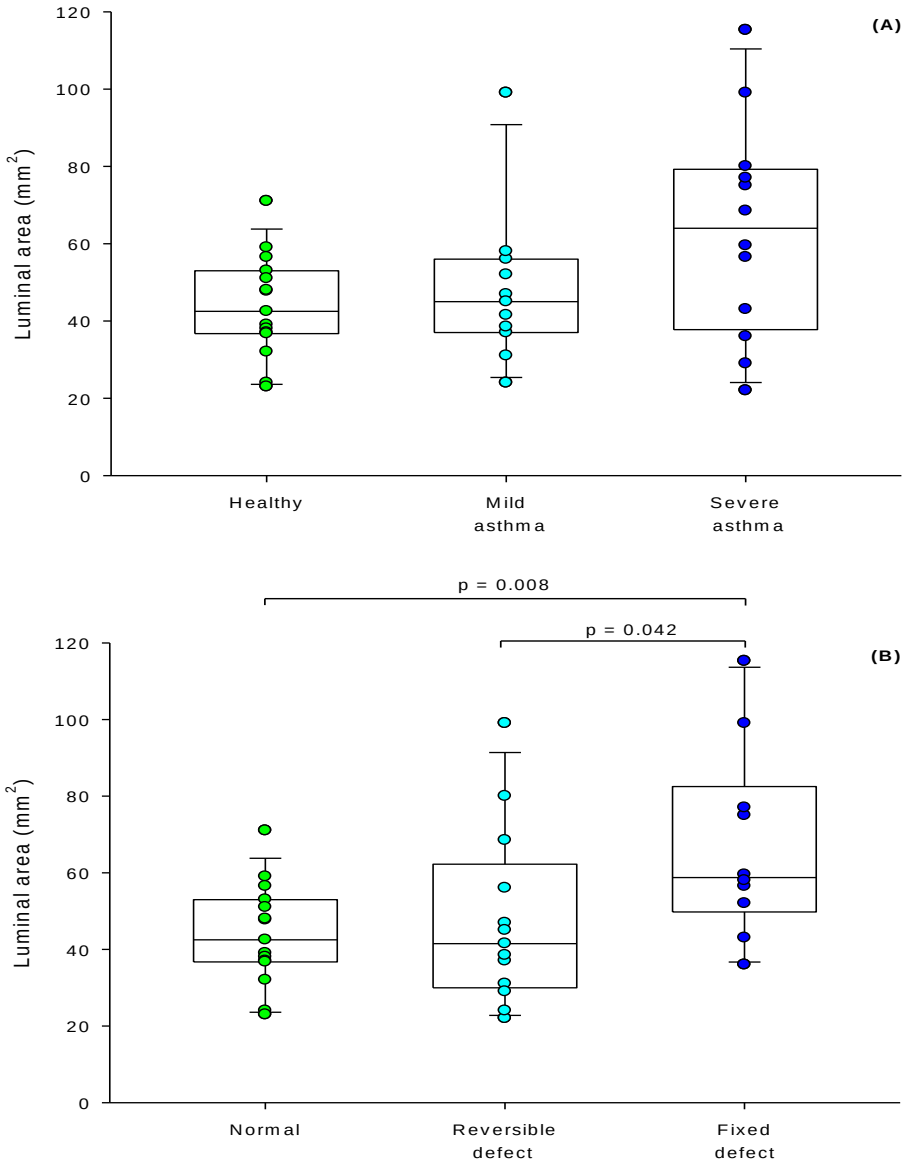

Figure 4.11. Luminal area by disease severity (A) and airflow defect (B).
(A) Subjects were divided into non-asthmatics (n=15), mild asthmatics (n=11) and severe asthmatics (n=12). Kruskal Wallis p = 0.152.
(B) Subjects were divided into those with normal airflow (n=15), reversible (n=13) and fixed (n=10) airflow defect. Kruskal Wallis p = 0.032.

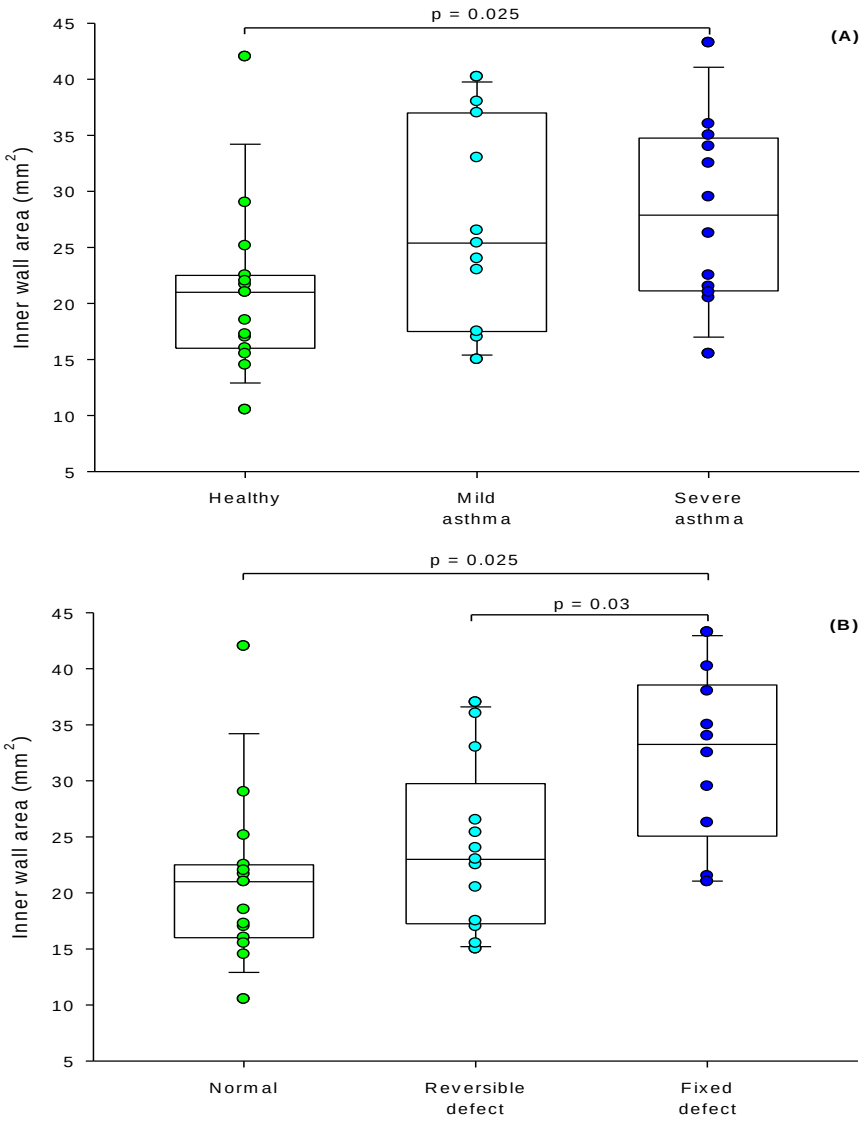

Figure 4.12. Inner wall area by disease severity (A) and airflow defect (B).
(A) Subjects were divided into non-asthmatics (n=15), mild asthmatics (n=11) and severe asthmatics (n=12). Kruskal Wallis p = 0.049.
(B) Subjects were divided into those with normal airflow (n=15), reversible (n=13) and fixed (n=10) airflow defect. Kruskal Wallis p = 0.006.

Normalisation of the inner wall area obviously depends on determining a ratio to a measure that is only varying as a function of the level of the airway where the readings are being taken. From the data shown above it is clear that luminal area, and consequently whole airway area, is therefore not a suitable parameter for normalisation. Luminal area could be changing with site of reading or potentially size of subjects. Considering the random nature of these factors, it seems very unlikely that they would vary with the nature of the disease and more probable that a true disease-dependent difference is being measured.

4.6.4 Correlations

Correlations between the inner wall area and physical indices of asthma were sought.

Baseline FEV_1 % predicted had a significant negative correlation with the inner wall area for all subjects. This was less evident if only the asthmatics were taken into account (Figure 4.13).

At least one HRCT study [205] suggests a link between airway wall thickness and asthma duration. According to the data presented here, there is a trend for thicker walls with longer duration of asthma. There is also a trend for the inner wall area to increase with the proportion of disease duration on inhaled corticosteroids (Figure 4.14). However neither of these parameters reaches statistical significance with the numbers studied.

As is the case with FEV_1 %, correlation of inner wall area with a measure of airway inflammation, exhaled nitric oxide (FeNO), shows significance only when all subjects are taken into account but not within the asthmatic group (Figure 4.15).

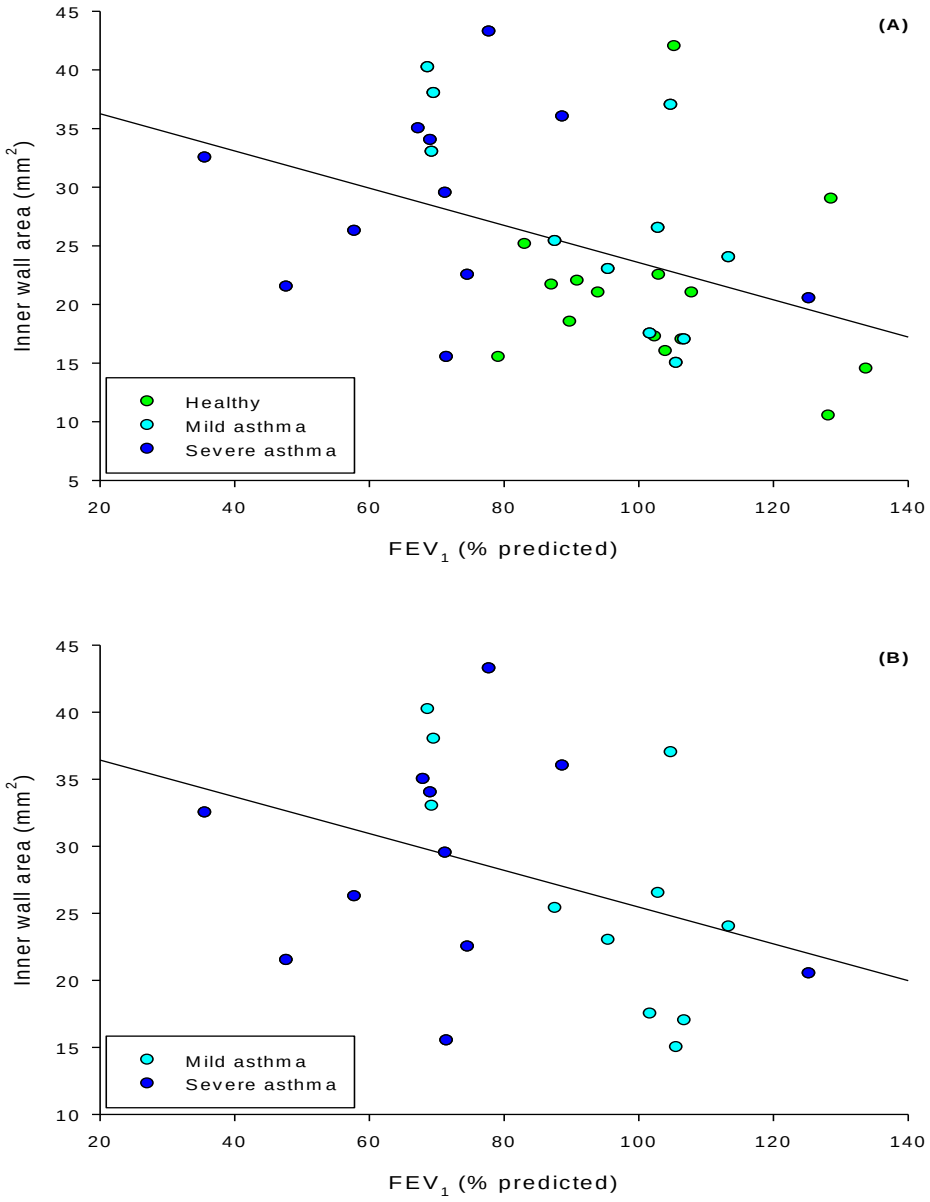

Figure 4.13. Correlation of inner wall area with FEV_1 % predicted in all subjects (A) and in asthmatics (B).

(A) Pearson correlation coefficient r(37) = −0.414; p = 0.011. r^2 = 0.17.

(B) Pearson correlation coefficient r(22) = −0.367; p = 0.092. r^2 = 0.13.

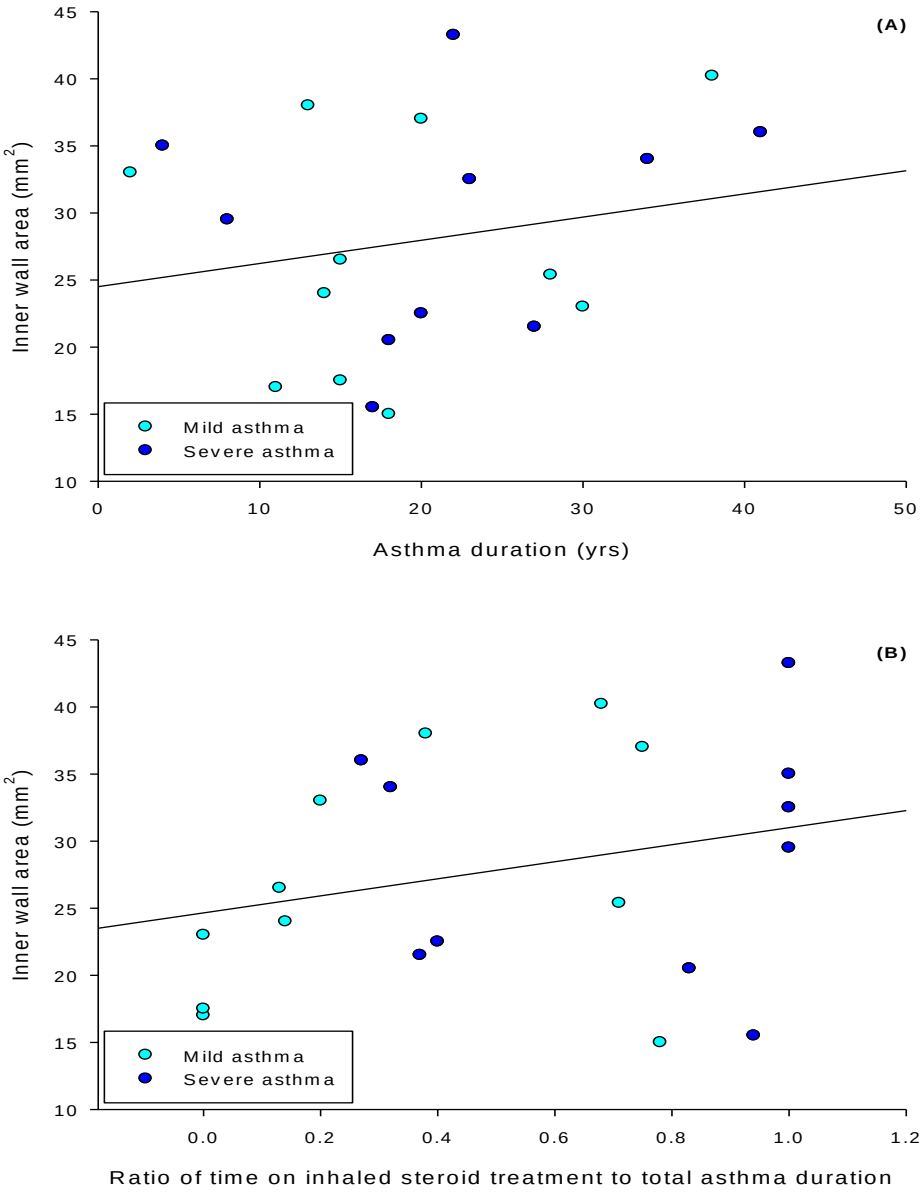

Figure 4.14. Correlation of inner wall area with asthma duration (A) and proportion of disease duration on inhaled corticosteroids (B).
(A) Pearson correlation coefficient r(21) = 0.207; p = 0.368. r^2 = 0.043.
(B) Pearson correlation coefficient r(21) = 0.272; p = 0.232. r^2 = 0.074.

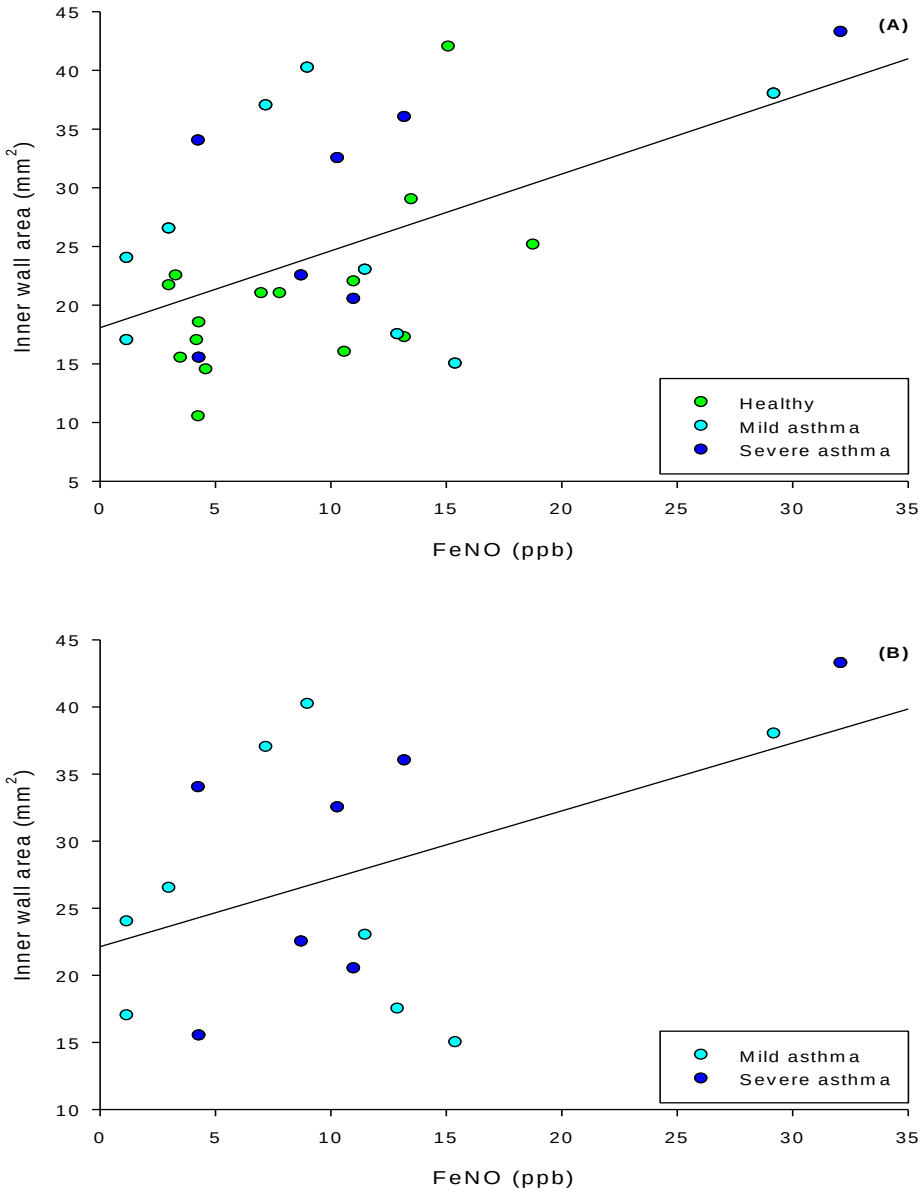

Figure 4.15. Correlation of inner wall area with exhaled nitric oxide in all subjects (A) and in the asthmatics (B).

(A) Pearson correlation coefficient r(31) = 0.523; p = 0.003. r^2 = 0.274.

(B) Pearson correlation coefficient r(16) = 0.469; p = 0.067. r^2 = 0.22.

In view of the unexplained variation in luminal area, a number of subjects were recalled for static lung volume measurement. It was hypothesised that the gas-trapping that is known to occur in more severe asthma there would be higher lung volumes which could affect luminal area through radial traction on the airway wall. Not all subjects were contactable but the available data seem to suggest a trend for correlation between the luminal area and residual volume post-bronchodilation (Figure 4.16).

In view of the small numbers in whom static lung volumes could be obtained as well as the significant time interval between the endobronchial ultrasound and the body plethysmography, correlation with baseline FVC percent of predicted was also assessed. FVC is determined by TLC and the fraction of TLC that can be exhaled forcibly – FVC percent predicted will therefore be reduced if TLC is reduced or air trapping is present. Sorkness *et al* [362] have shown that FVC percent predicted can be used as a surrogate marker of air trapping in asthma. Luminal area showed a trend for negative correlation with baseline FVC percent of predicted in the asthma group (Figure 4.17).

Neither of these correlations reaches statistical significance.

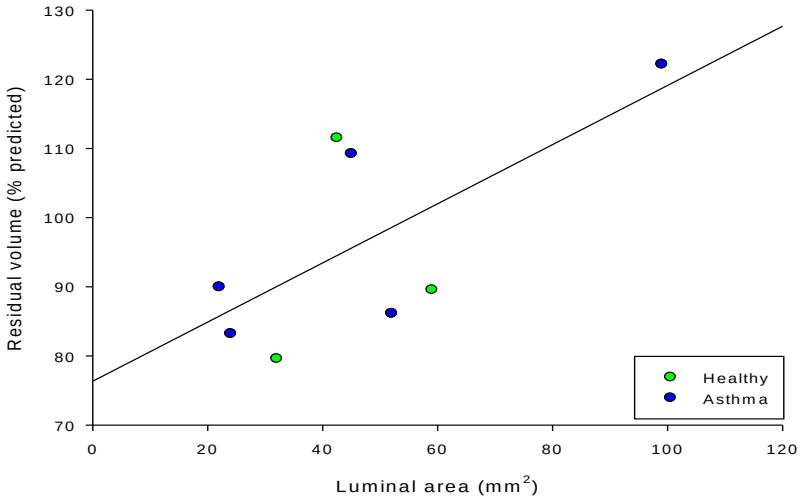

Figure 4.16. Correlation of luminal area as measured by EBUS and residual lung volume (RV) percent of predicted post bronchodilation with nebulised salbutamol (2.5mg).

Spearman correlation coefficient r(9) = 0.429; p = 0.289. r^2 = 0.458.

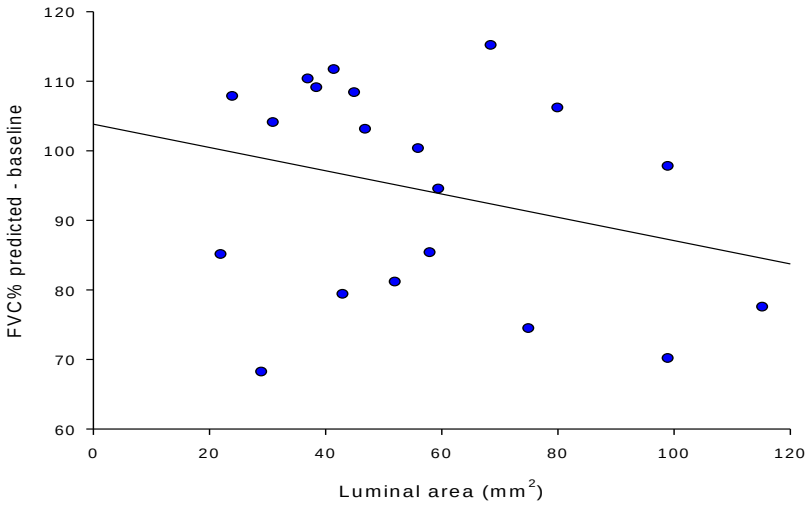

Figure 4.17. Correlation of forced vital capacity percent of predicted with luminal area in asthmatic subjects.

Pearson correlation coefficient r(20) = -0.287; p = 0.22. r^2 = 0.082.

Due to clinical considerations, a number of subjects do not have airway responsiveness data. Although an assessment of airway hyperresponsiveness was scheduled for all subjects taking part in this study, many of the severe asthmatics had a low baseline FEV_1 that precluded performance of this test. Data was available for the non-asthmatics who all had PC_{20} to methacholine above 16 mg/ml. In the 13 asthmatics for whom PC_{20} was available, most of whom had relatively milder asthma, there were two distinct groups – those with hyperreactive airways (PC_{20} <8 mg/ml) and those with non-reactive airways (PC_{20} >16 mg/ml). When their PC_{20}s are plotted there is a noticeable tendency for the subjects with hyperreactive airways to have thinner inner wall areas (Figure 4.18).

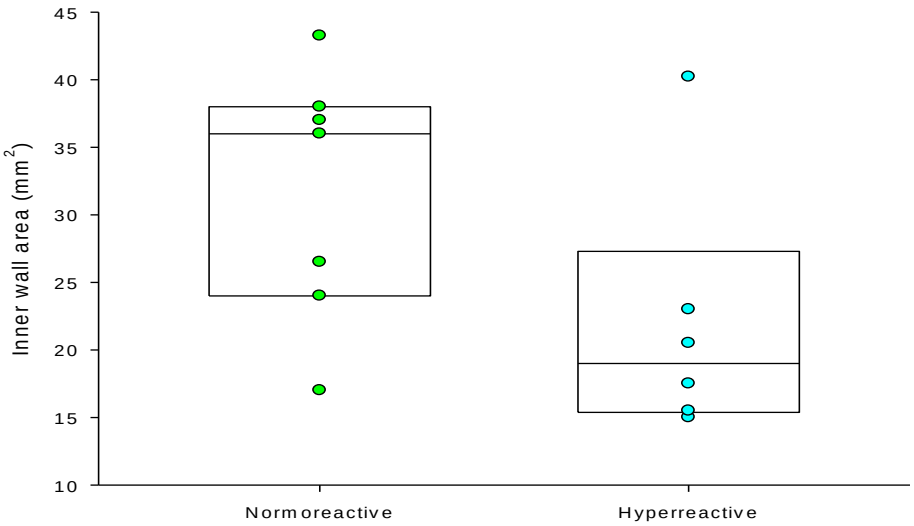

Figure 4.18. Inner wall area by airway reactivity.
Methacholine bronchial challenge data available for 13 asthmatics, divided into 7 with normoreactive airways having a PC_{20} >8mg/ml (5 mild asthmatics and 2 severe asthmatics) and 6 with hyperreactive airways having a PC_{20} <8mg/ml (4 mild asthmatics and 2 severe asthmatics). Mann Whitney U p = 0.101.

An increase in the reticular basement membrane thickness is one of the hallmarks of remodelling. Endobronchial biopsies taken at the same bronchoscopy were stained with Toluidene Blue [30] and the average thickness of the reticular basement membrane measured with the aid of computer software (Zeiss KS 400 image analysis system, Welyn Garden City, UK). There was no significant correlation with inner wall area as measured by endobronchial ultrasound (Figure 4.19). Neither was there any difference in reticular basement membrane thickness between asthmatics and non-asthmatics.

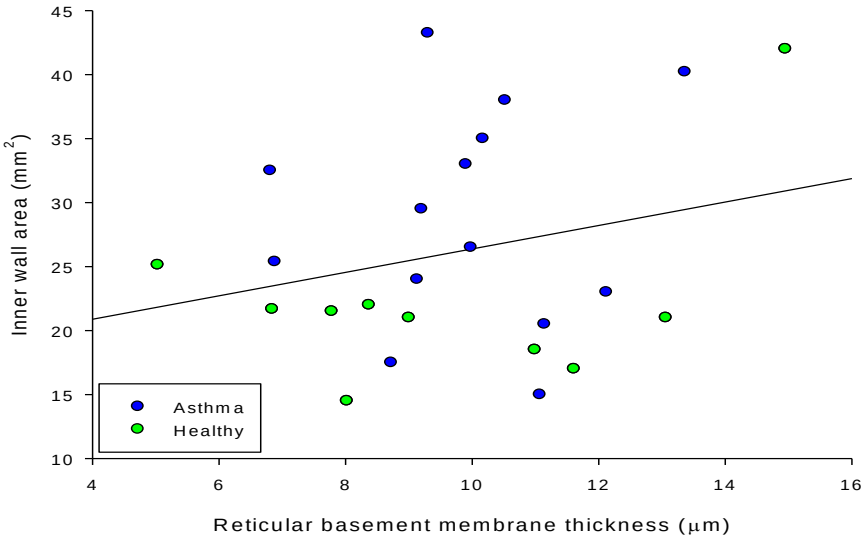

Figure 4.19. Correlation of reticular basement membrane thickness with inner wall area.
Pearson correlation coefficient r(24) = 0.249; p = 0.241. r^2 = 0.062.

4.7 Discussion

These data suggest that in asthma there is an increase in the thickness of the airway, specifically in the epithelial, submucosal and smooth muscle

compartments. This corroborates the evidence from post-mortem studies. On the other hand, most HRCT evidence also points towards thickening of the airway wall but that modality lacks the ability to distinguish the different layers of the bronchial wall. So this can be considered as in vivo evidence of the compartmental thickening alluded to in post-mortems.

Interpretation of ultrasonographic images entails a considerable degree of subjectivity in view of the limited resolution of the technology. These images were analysed by one person, blinded as to the status of disease, over a relatively short span of time. This was done in order to increase cohesion when performing the measurements.

The inner wall area is measured from ultrasound videos with a number of potential sources of error. An arbitrary decision is taken in choosing the still images to capture from the video and use in subsequent analysis – an attempt was made to try to determine the point combining best image with least airway distortion. Resolving where to define the boundary between the different echo bands is usually not clear cut. Consistency in taking these parameters, as well as having done the measurements in more than one image and then averaging may mitigate some of these potential pitfalls.

The validated measures used in CT studies, namely the T/D ratio and the PWA are ratios rather than absolute values. This normalises the wall thickness measure to a total airway parameter and eliminates any possible bias from physiological differences in airway size [201]. When this was attempted in the ultrasound measure of IWA, the differences noted in the different groups was abrogated. On further scrutiny, this was found to be the result of a difference in the luminal area of the asthmatics versus the non-asthmatics. In fact asthmatics with a fixed airflow defect had an average luminal area of 67.1 (36 – 115.3) mm^2; the asthmatics with a reversible airflow defect had a mean of 47.6 (22 – 99) mm^2 and

the non-asthmatics 43.9 (23 – 71) mm^2. Kruskal Wallis analysis of the data showed a significant difference among the groups with a p value of 0.032.

There is no documented data in the literature describing an increased airway luminal diameter as a feature of asthma. A number of the CT studies [214, 363, 364] have found increased bronchial dilatation in asthmatic scans compared to controls. However this is not based on direct measurement but rather on a radiological phenomenon referred to as the 'signet ring sign' where bronchial dilatation results in an increase in size of the airway such that its diameter is more than that of the accompanying bronchial artery. Direct measurement of luminal area from CT images by Okazawa *et al* revealed no difference between asthmatics and healthy controls, although the 6 asthmatics in this study had milder asthma treated only with β_2-agonists [202].

In another study [365], using relatively small numbers of subjects, a probe was inserted into the bronchial tree at various levels down to distal bronchus intermedius where measures of the luminal area and bronchial wall compliance were made. The investigators found no difference between asthmatics and non-asthmatics, the only significant variation in the luminal area being found between males and females – the latter having smaller areas than the former. There was no significant difference in our cohort's luminal area between males and females.

Another possible reason for the increased luminal dimensions in asthmatics could be easier distensibility of the bronchial wall. Since the ultrasonographic method being used involves inflation of a water-filled balloon around the probe to achieve sonic coupling, there is the risk that the lumen is being artificially expanded by the procedure itself. This is unlikely to be the case as all the evidence in the literature [365] points to asthmatic airways being less, rather than more compliant.

Asthma, particularly more severe asthma, is associated with some degree of air trapping [362] and it is possible that the dilation of the bronchial lumina is a consequence of traction on the airways from the surrounding hyper-expanded lung. The potential correlation with residual volume and forced vital capacity could point to this possibility.

The correlations that were found using this ultrasonographic assessment reflect what is reported in the CT studies. The small numbers probably account for the weakness in correlation in most cases.

There was a significant negative correlation with FEV_1 % reflecting a difference between asthmatics and non-asthmatics rather than a linear relationship within the disease range of severity. This also seems to hold true when trying to equate wall thickness with a measure of ongoing inflammation.

Airway hyperresponsiveness is particularly interesting. Its relationship with airway wall thickness is still contentious – some studies showing a positive whilst others show a negative correlation. The data presented here, although admittedly lacking in power, would suggest a protective effect of the increased smooth muscle and submucosal layers. This would tie in with data from Niimi *et al* [143] where increased whole wall thickness was negatively related with airway reactivity. Their measurement of hyperresponsiveness to methacholine involved continuous monitoring of airway resistance rather than determining a 20% drop in FEV_1 by intermittent spirometry as was done in the data presented above. This allowed distinction between airway sensitivity, the point when airway resistance started to increase and airway reactivity, the slope of the resistance curve reflecting the rate of change of airway resistance. Airway wall thickness correlated negatively with this latter measure.

Reticular basement membrane correlation has been particularly disappointing. Numerous studies have shown asthmatics to have increased subepithelial fibrosis

which results in thickening of the reticular basement membrane [65, 88]. A large study including 50 oral steroid-treated severe asthmatics has indicated that it is an effective differentiating feature of severe asthma [366]. In the samples analysed here, a difference in thickness between asthmatics and non-asthmatics was not evident suggesting that this may be a less reliable criterion in this group of subjects.

The data presented in this chapter lends legitimacy to the idea of dividing the recruited subjects into 'thick' and normal airways. Determining the cut-off point at which wall thickness crosses the boundary between physiology and pathology would obviously need much larger numbers than was undertaken here. This would seem a more sensible way of trying to determine differences in pathophysiology than asthma severity which is a less objective measure. By inference fixed airflow obstruction via a post-bronchodilator FEV_1 can be used as a substitute for airway wall with a thickened submucosa.

CHAPTER 5

In Vitro Assessment of Output of Extracellular Matrix Proteins by Primary Fibroblasts from Central Airways with Different Degrees of Remodelling

5.1 Proteoglycans

5.1.1 General

5.1.1.1 Structure

Proteoglycans are heavily glycosylated glycoproteins which are ubiquitous in most tissues of the human body. They consist of a protein core to which are covalently attached a number of glycosaminoglycan chains.

They are a very heterogeneous group of molecules – the protein core can vary between 10 kDa to >500 kDa and the number of glycosaminoglycan chains attached can be anything from one to more than a hundred.

Gycosaminoglycans (GAGs) are long unbranched chains of repeating disaccharide units consisting of a hexuronic acid or hexose alternating with a hexosamine, typically in a sequence of about 40 – 100 repeats. The most common GAGs (with their disaccharide units in brackets) are: chondroitin sulphate (glucuronic acid, N-acetylgalactosamine), dermatan sulphate (glucuronic or iduronic acid, N-acetylgalactosamine), keratan sulphate (galactose, N-acetylglucosamine) and heparin or heparan sulphate (glucuronic or iduronic acid, N-acetylglucosamine). The sulphation of these chains can be controlled and determines their function. Varying numbers and types of GAGs can bind to about 20 genetically different protein cores giving a large group of proteoglycans with very diverse functions [196].

5.1.1.2 Classification

The classification of proteoglycans is still a matter of some contention. Various ways of classifying proteoglycans are described in the literature – based on structure, distribution or a combination of the two.

Cell-associated proteoglycans, such as syndecans and glypicans are mainly found on the cell surface although they can also occur within the cytoplasm and may even translocate to the nucleus. They mainly cover roles as co-receptors for growth factors, in cell adhesion and differentiation [367].

Matrix proteoglycans are of more relevance to asthma remodelling. They can be broadly divided into three sub-groups.

Basement membrane-associated proteoglycans include perlecan, agrin and bamacan. They are structurally and functionally heterogeneous. Perlecan appears to be involved in angiogenesis, growth factor regulation and renal tubular epithelial permeability. Agrin is mainly found in synaptic basement membranes whilst bamacan, which is widely distributed, is yet to be assigned a clear role [196].

Small leucine-rich proteoglycans are characterised by relatively low molecular weights and a central leucine-rich domain. They are mostly implicated in collagen fibrillogenesis [368].

Hyalectans or lecticans are large proteoglycan molecules with three main domains – an N-terminal hyaluronan-binding domain, a glycosaminoglycan binding-domain and a C-terminal lectin-binding domain. Aggrecan, versican, neurocan and brevican are the members of this class [196].

5.1.1.3 Proteoglycan synthesis and regulation

Different tissues will contain different types and proportions of proteoglycans. Fibroblasts, vascular smooth muscle cells, endothelial cells, as well as epithelia have all been shown to express proteoglycans. Proteoglycan synthesis involves the formation of a core protein to which a tetrasaccharide (xylose-galactose-galactose-glucuronic acid) is attached via a serine residue in the protein through

the action of a xylosyltransferase. Within the Golgi apparatus, repeating disaccharides are added sequentially to the linker tetrasaccharide, giving rise to the GAG chain. Part of the information that directs GAG synthesis is coded within the core protein itself. After sulphation of the glycosaminoglycan chains, the formed proteoglycans are either secreted (e.g. aggrecan, decorin, biglycan), transported to the cell membrane (e.g. syndecans) or shifted to the cytosolic compartment (e.g. serglycins) [369]. Although not much is known about proteoglycan breakdown, it is thought that there is a continuous turnover of these matrix components, with various metalloproteinases, hyaluronidases and other enzymes mediating the degradation [370].

5.1.1.4 Functions

Proteoglycans of the extracellular matrix play active roles within their micro-environment. The large negatively-charged glycosaminoglycan chains can fill space, bind water molecules and repel negatively charged molecules. Proteoglycans can bind to a number of proteins, either sequestering them to prevent untimely interactions or facilitating their spatial proximity with substrate to allow interactions to occur. They can bind to growth factors, proteases and anti-proteases, extracellular matrix proteins and cell-surface proteins. In this way they are involved in cell signalling, cell adhesion and migration and extracellular matrix assembly [196].

In this chapter emphasis is placed on the matrix proteoglycans. The main airway basement membrane proteoglycan is perlecan and there is evidence of its increased production in asthma [371]. It modulates basement membrane properties and binds growth factors, proteinases and anti-proteinases with particular relevance in angiogenesis [196].

5.1.2 Small leucine-rich proteoglycans

Small leucine-rich proteoglycans (SLRPs) are characterised by a central leucine-rich domain flanked by cysteine-rich clusters on either side. Members of this class of proteoglycans include decorin, biglycan, lumican, keratocan, fibromodulin and epiphycan. Keratocan is confined to the corneal extracellular matrix whilst epiphycan is localised to cartilage growth plates. Although present in the lung, fibromodulin does not appear to be prevalent in the airways [130]. The potential players in asthma remodelling are therefore decorin, biglycan and lumican.

5.1.2.1 Decorin

Decorin has a 36 kDa core protein molecule consisting of the group-defining central leucine-rich repeat sequence with cysteine-rich clusters on either side. Four domains are identified in its structure (Figure 5.1). The first domain consists of a signal peptide and a propeptide. The former is responsible for directing the protein to the rough endoplasmic reticulum, whilst the function of the propeptide is less obvious. When the propeptide is deleted, decorin forms shorter glycosaminoglycan chains, suggesting a role in the glycosaminoglycan chain synthesis. The second domain consists of a cysteine-rich region to which the GAG chain is attached – dermatan or chondroitin sulphate depending on the tissue of origin. Domain 3 is made up of 10 tandem repeats of leucine-rich regions (LRR) where binding to other proteins occurs. In this area, decorin has 3 potential sites of substitution of the protein core with N-linked oligosaccharides which can modulate its protein interactions. The LRR gives decorin a horse-shoe shape which proffers a concave surface for interaction with ligands. The fourth domain is another cysteine-rich area with unclear functions [196]. One possibility is that it enables the protein to form a loop through a disulphide bond, which appears to be important for its interaction with collagen [372].

Decorin's functions include control of collagen fibrillogenesis, modulation of cell adhesion and growth factor activity [373].

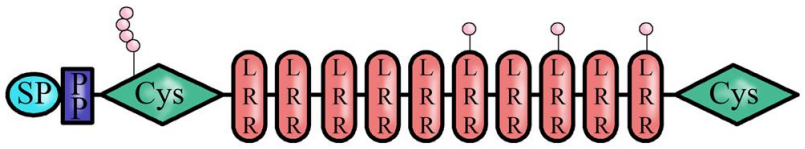

Figure 5.1. Schematic representation of the structure of Decorin.

Evidence from mice with targeted disruption of decorin suggests that it is important for organised collagen production. In the absence of decorin, the fibrils are irregular and haphazard [374]. It is postulated that the fibrils forming from tri-helical collagen molecules by lateral association fit within the concavity of the horse-shoe shape of decorin which maintains the appropriate diameter by forming anti-parallel duplexes between the GAG chains of decorins on adjacent fibrils [375].

Decorin appears to mostly inhibit cell proliferation and spread through inhibition of growth factors including TGFβ and EGF [376, 377]. It also appears to have a direct effect on growth suppression through upregulation of p21 in a TGFβ-independent fashion [378].

5.1.2.2 Biglycan

Biglycan has a high degree of homology in its structure with decorin (Figure 5.2). In contrast to decorin, it is associated with two chondroitin or dermatan sulphate GAG chains at the N-terminal end of the core protein. It also has potential for two

rather than three N-linked oligosaccharide substitutions within the cysteine-rich domain.

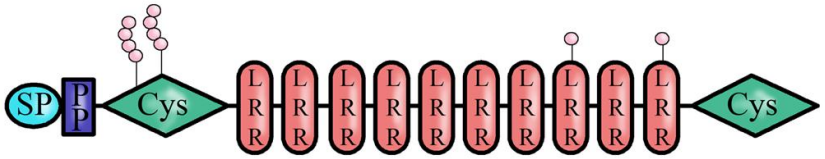

Figure 5.2. Schematic representation of the structure of Biglycan.

Biglycan's functions have been studied less than those of decorin. It appears to interact in a similar fashion with collagen fibrils during their formation, possibly even competing with decorin for the same binding sites [379]. Collagen fibrils in the tendons of biglycan-deficient mice were smaller in diameter than wild-type [380].

It is also capable of binding TGFβ, sequestering it in the extracellular matrix [381], and may be capable of compensatory upregulation in the absence of decorin [382].

One study tracing the expression of decorin and biglycan during the course of mouse tendon development showed peaking of decorin in the early phases of fibrillogenesis which then decreases to a stable plateau. In contrast, biglycan decreases quickly to negligible levels. In the same experimental set-up, decorin-deficient mice showed upregulation of biglycan presumably in compensation for the decorin absence – although the compensation is never complete and abnormalities in the fibrils accumulate, particularly in the later stages of fibrillogenesis, when biglycan levels would be low in the wild-type mice [383].

This suggests that the sequential expression of these SLRPs may be particularly important.

5.1.2.3 Lumican

Lumican differs from the previous two SLRPs by lacking a propeptide and having the possibility of binding 3 to 4 keratan sulphate GAG chains in specific locations in the central leucine-rich region (Figure 5.3). These chains are reported to be highly sulphated in the cornea but are poorly- or non-sulphated in other tissues.

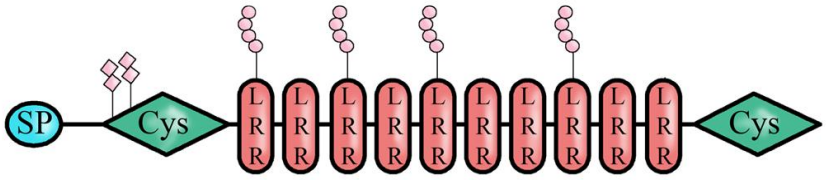

Figure 5.3. Schematic representation of the structure of Lumican.

Lumican has been mostly studied in the cornea, hence its name, where the orderly organisation of collagen fibrils is fundamental to the maintenance of transparency. Studies with lumican knock-out mice show a similar effect to deficiencies of the other SLRPs – disorganised collagen fibrils which appear to be on average larger in diameter [384]. Lumican binds to different sites on the collagen molecule than decorin or biglycan. It has a high degree of homology with fibromodulin, another SLRP, which uses the same binding site on collagen [385]. Its role in growth control has not been extensively studied.

5.1.3 Hyalectans

Hyalectans are characterised by their hyaluronan binding capabilities through one of the domains of the tri-domain structure. They are larger than the SLRPs, with the molecular weights of their core protein in the range of 200 kDa. The main role they cover is that of a structural proteoglycan, particularly the prototype aggrecan. The other members are versican, brevican and neurocan. Only versican appears to be relevant to the inner bronchial wall, aggrecan being mostly confined to the cartilage. Brevican and neurocan are important proteoglycans in the central nervous system [196].

5.1.3.1 Versican

Versican, the largest member of the family, follows the tri-domain structural conformation of the rest (Figure 5.4). The first domain consists of an immunoglobulin-type repeat followed by two link protein modules which mediate binding to hyaluronan. The second domain consists of 2 regions designated GAG-α and GAG-β which have up to 30 potential attachment sites for chondroitin sulphate chains and numerous N- or O-linked oligosaccharides. Four possible splice variants of versican are known to occur – V0 contains both GAG-α and GAG-β, V1 contains only GAG-β, V2 has just the GAG-α and V3 has neither. These can bind different numbers of GAG chains and in fact none n the case of the V3 variant. Different cell types appear to express different variants. Domain three includes two EGF-like repeats, a C-type lectin domain and a complement regulatory protein-like module. This is thought to be responsible for binding to less complex sugars such as lectins [196].

Versican derives its name from the diversity of its functions. It mediates interaction between cell surface proteins and the extracellular matrix. It is generally thought of as being anti-adhesive via domain 1 [386], however in certain instances it can act in a pro-adhesive capacity via the third domain [387].

Increased versican levels are usually found in tissues where cells are undergoing proliferation, possibly to render the pericellular matrix more favourable to cell expansion. It appears to be capable of acting as a mitogen itself via the EGF-like sequences in the C-terminal domain [388]. A combination of its anti-adhesive and pro-mitogenic functions is probably important to promote growth [389].

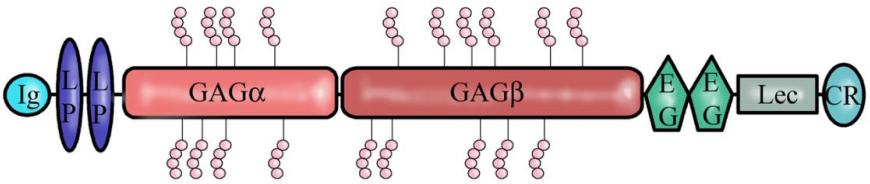

Figure 5.4. Schematic representation of the structure of Versican.

Versican interacts with a number of proteins of the extracellular matrix. Most studied is the binding to hyaluronan via its amino terminal domain. The resultant highly negatively-charged complex can bind water and confers turgor to the extracellular matrix. Changes in binding may thus impact on the size and physical characteristics of the extracellular matrix [373].

Versican interacts with tenascin-R, fibrillin and fibulin 1 and 2 through its C-terminal domain. Fibrillin and the fibulins are involved in elastic fibre assembly. The relative proportions of versican variants seems to be of importance here – for example it has been shown that the presence of the V3 non-GAG variant in higher proportions promotes the synthesis of elastic fibres [390].

5.1.4 Proteoglycans in asthma

A number of proteoglycans appear to be increased in asthma, including versican, biglycan, hyaluronan, perlecan and lumican.

Increased expression of proteoglycans in the airways of mild steroid-naïve asthmatics was shown in a study by Huang *et al* in 1991 [130]. Immunohistochemical staining of bronchial biopsies showed significantly higher quantities of lumican, biglycan and versican in the subepithelial layers of asthmatics (n=7) compared to healthy controls (n=6). Moreover, this was negatively correlated with a measure of airway hyperresponsiveness. This suggests a role for proteoglycans in the changes in airway dynamics that develop in asthma.

Assessment of proteoglycan production by fibroblasts retrieved from endobronchial biopsies showed increased proteoglycan production, at protein level, in cultures from the mild asthmatics with the more hyperresponsive airways [371].

More severe asthma is the disease state in which one would expect more remodelling and therefore potentially more changes in the extracellular matrix. In the case of proteoglycans the evidence here becomes more conflicting. Post-mortem immunohistochemistry on random lung biopsies from non-asthmatics and fatal cases of status asthmaticus (n=18) showed increased versican staining in the inner bronchial wall of small airways and reduced decorin and lumican in the outer wall in the latter [131]. Pini *et al* looked at proteoglycan immunostaining in endobronchial biopsies of moderate and severe asthmatics. They found increased deposition of biglycan and lumican in the smooth muscle layer of the moderate asthmatics [132]. The authors postulate that the increased proteoglycan

in the smooth muscle may be playing a protective role and thereby modulate disease severity.

Two of the recurring limiting factors in such studies are the significant degree of subjectivity in defining asthma severity and the absence of a single universally accepted hallmark of remodelling.

5.2 Aims

Proteoglycans are increased in asthma with one study [371] suggesting that fibroblasts derived from asthmatic airways secrete more proteoglycans. Correlation with severity is less clear, partly due to the difficulties in asthma classification. The aim of these experiments is to determine differences in trends of in vitro proteoglycan expression at baseline and in response to a pro-inflammatory cytokine (TNFα) of particular relevance in severe asthmatics, and a pro-fibrotic cytokine (TGFβ), dividing the subjects on the basis of airflow defect.

The hypothesis is that there is a difference in proteoglycan synthesis between primary fibroblasts derived from asthmatics with a fixed airway obstruction and those with a reversible defect.

5.3 Methods

5.3.1 Subject characteristics

Fibroblast cultures from the subjects whose characteristics are summarised in Table 5-1 were used in these experiments.

Table 5-1. Subject characteristics.

	Disease	No.	Age* (yrs)	Sex (F:M)	FEV_1 pre-BD* (% predicted)	ICS* (mcg/day BDP equivalent)
Unstimulated fibroblasts	Normal	10	42.2 (23-64)	6 : 4	108 (90-134)	0
	Reversible	11	36.2 (22-64)	7 : 4	94 (69-113)	218 (0-1000)
	Fixed	10	54.7 (42-70)	7: 3	55 (30-70)	2589 (640-7200)
TNFα treated fibroblasts	Normal	2	20.5 (20-21)	0 : 2	112 (112)	0
	Reversible	3	34 (21-45)	2 : 1	103 (96-111)	0
TGFβ treated fibroblasts	Healthy	4	51.3 (38-64)	2 : 2	115 (90-134)	0
	Reversible	5	37.8 (26-64)	4 : 1	94 (69-113)	240 (0-1000)
	Fixed	4	48 (42-54)	2 : 2	54 (30-70)	1950 (800-3600)

*Data shown as mean with range in brackets.
FEV_1 pre-BD = forced expiratory volume in 1 second as percentage predicted prior to bronchodilation.
ICS = inhaled corticosteroid, BDP = beclometasone dipropionate.

5.3.2 Baseline fibroblast proteoglycan mRNA

Primary fibroblasts from non-asthmatics (n=10), mild asthmatics with a reversible airflow defect (n=11) and severe asthmatics with a fixed airflow defect (n=10) were cultured as previously described.

They were plated on 12-well plates previously coated with collagen 1 and allowed to grow to subconfluence. They were then serum-starved for 24 hours after which Trizol™ was applied to the wells for RNA extraction. RNA extraction, reverse transcription and RT-PCR were performed as described elsewhere. Relative

quantification of mRNA expression for decorin, biglycan, lumican and versican was performed on the cDNA obtained. mRNA levels were measured using primers for DCN (decorin), BGN (biglycan), LUM (lumican) and VCAN (versican) (PrimerDesign, UK) via real-time qPCR (iCycler, BioRad). Values were normalised to reference genes phospholipase A2 (A2) and ubiquitin C (UBC) [391].

For a subset of these subjects, data on airway wall thickness from ultrasonographic measurements were available. This allowed correlation between proteoglycan expression and bronchial wall dimensions.

5.3.3 Stimulated fibroblast proteoglycan output

In order to determine response to stimulation by cytokines, primary fibroblast cultures were exposed to a pro-inflammatory cytokine, TNFα, and a pro-fibrogenic cytokine, in the form of TGFβ.

It is quite difficult to determine an appropriate dose to use for stimulating primary fibroblasts. Levels documented in studies using bronchoalveolar lavage were mostly looked at. Bronchoalveolar lavage concentrations, however, are at best a surrogate for the levels that would actually be found in the bronchial wall in close proximity to the airway wall fibroblasts. There is a degree of dilution in bronchoalveolar lavage fluid and the polarised epithelial cells can have differing expression of mediators from their apical and basal surfaces. TNFα concentration in BALF has been quoted as anything between 0 and 3500pg/ml in asthmatic airways [392-394], with stable, post-bronchial challenge and post-status asthmaticus subjects studied. For TGFβ, values between 0 and 7ng/ml have been found [394-396] although assays vary as to whether total TGFβ or specific isoforms are being measured. $TGF\beta_2$ was chosen due to the fact that it is the main isoform expressed by epithelial cells after injury [397].

5.3.3.1 mRNA experiments

Fibroblasts were grown in collagen-coated 12-well plates and stimulated with serum-free medium and increasing doses of TNFα (1 ng/ml and 10 ng/ml) and TGFβ (1 ng/ml and 10 ng/ml) for 24, 48 and 72 hours. RNA was extracted at each time-point and reverse-transcribed to cDNA as previously described.

Proteoglycan expression in these samples was assessed via qPCR.

5.3.3.2 Protein experiments

For TGFβ-treated cells, plates for protein quantification were set up concurrently with those for mRNA measurement. Collagen-coated 96-well plates were seeded with fibroblasts, and then serum-starved for 24 hours once they had reached sub-confluence. They were then treated with serum-free medium or $TGF\beta_2$ (1 ng/ml and 10 ng/ml) applied in rows for 24, 48 and 72 hours. At the respective time-point, the wells were washed with PBS and then the cells were removed by applying hypotonic (0.02M) NH_4OH for 5 minutes. This method described for smooth muscle cell cultures by Johnson *et al* [398] disrupts the cell layer and leaves the extracellular matrix behind. The wells were then washed with PBS, sealed and stored at -70°C with a few µl of PBS in each well for cover until used for ELISA quantification of protein.

A semi-quantitative ELISA was performed on the thawed plates with biotinylated antibodies against decorin and versican. The wells were first washed with 0.05% Tween-20, then blocked for 60 minutes with 1%BSA and washed, after which the biotinylated anti-proteoglycan antibody was applied for 120 minutes. After washing, Streptavidin-HRP (Biosource International) was added to the wells for 30 minutes and excess washed off at the end. The substrate, tetramethylbenzidine (TMB Single solution, Invitrogen), was pipetted into the plates and colour development allowed to occur over about 10 minutes. The reaction was stopped

with 2M H_2SO_4. Optical densitometry was then measured at 650nm on an ELISA plate reader (Multiskan Ascent, Thermo Fisher Scientific, US). Since the ELISA did not include a standard curve, values were normalised to an index plate with matrix from unstimulated fibroblasts and which was used at each ELISA run.

5.3.4 Statistics

Non-parametric statistical analysis (Kruskal Wallis, Mann-Whitney U) was used to compare differences between the groups. Correlations were explored using Spearman's correlation coefficient. A p value of <0.05 was taken to denote statistical significance.

5.4 Results

5.4.1 Baseline fibroblast proteoglycan mRNA

No statistically significant differences were found in the basal expression of biglycan (Figure 5.5 A), decorin (Figure 5.5 B), lumican (Figure 5.5 C) and versican (Figure 5.5 D) by the quiescent fibroblast cultures.

Neither was there any significant correlation with the inner wall area (IWA) thickness as measured by EBUS in a subset of these subjects (Figure 5.6 and Figure 5.7).

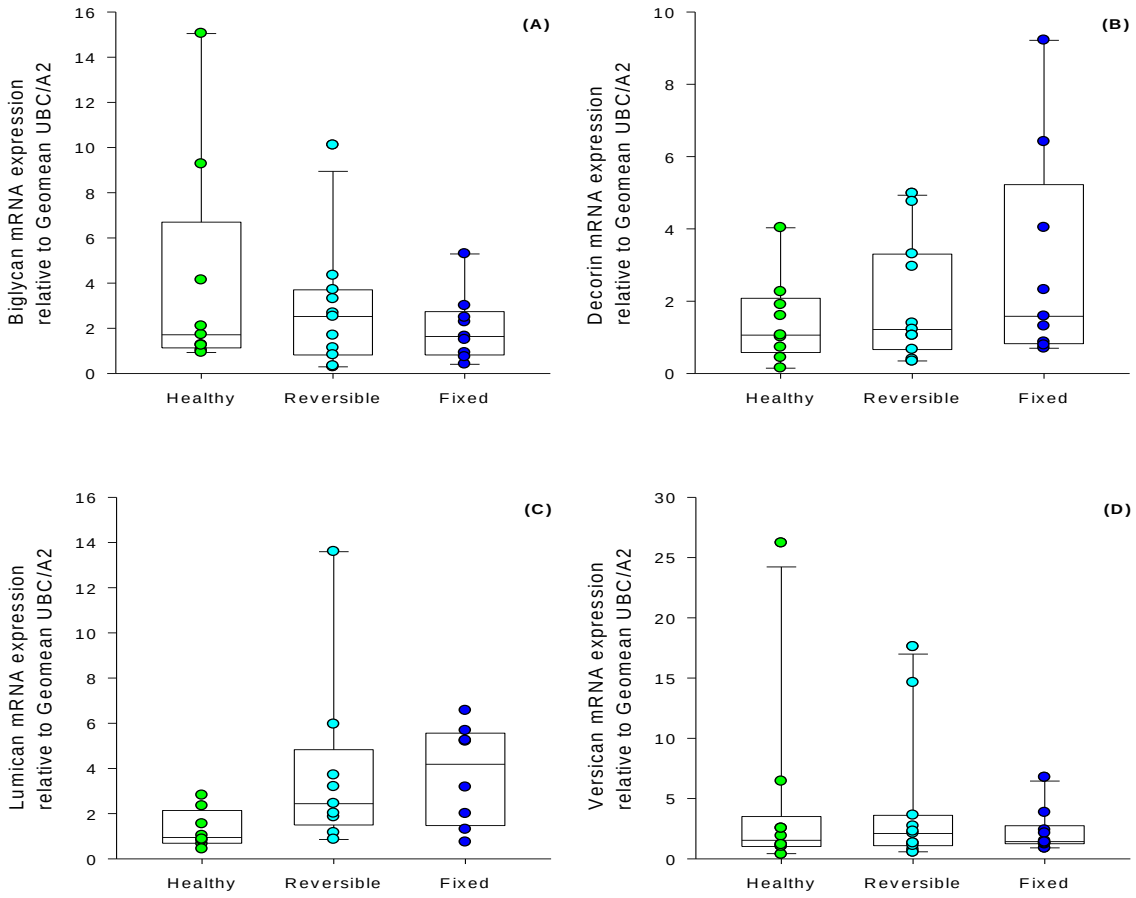

Figure 5.5. Expression of Biglycan (A), Decorin (B), Lumican (C) and Versican mRNA (D) in unstimulated fibroblasts.

All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratio between fibroblasts from asthmatic and non-asthmatic subjects = 0.997.

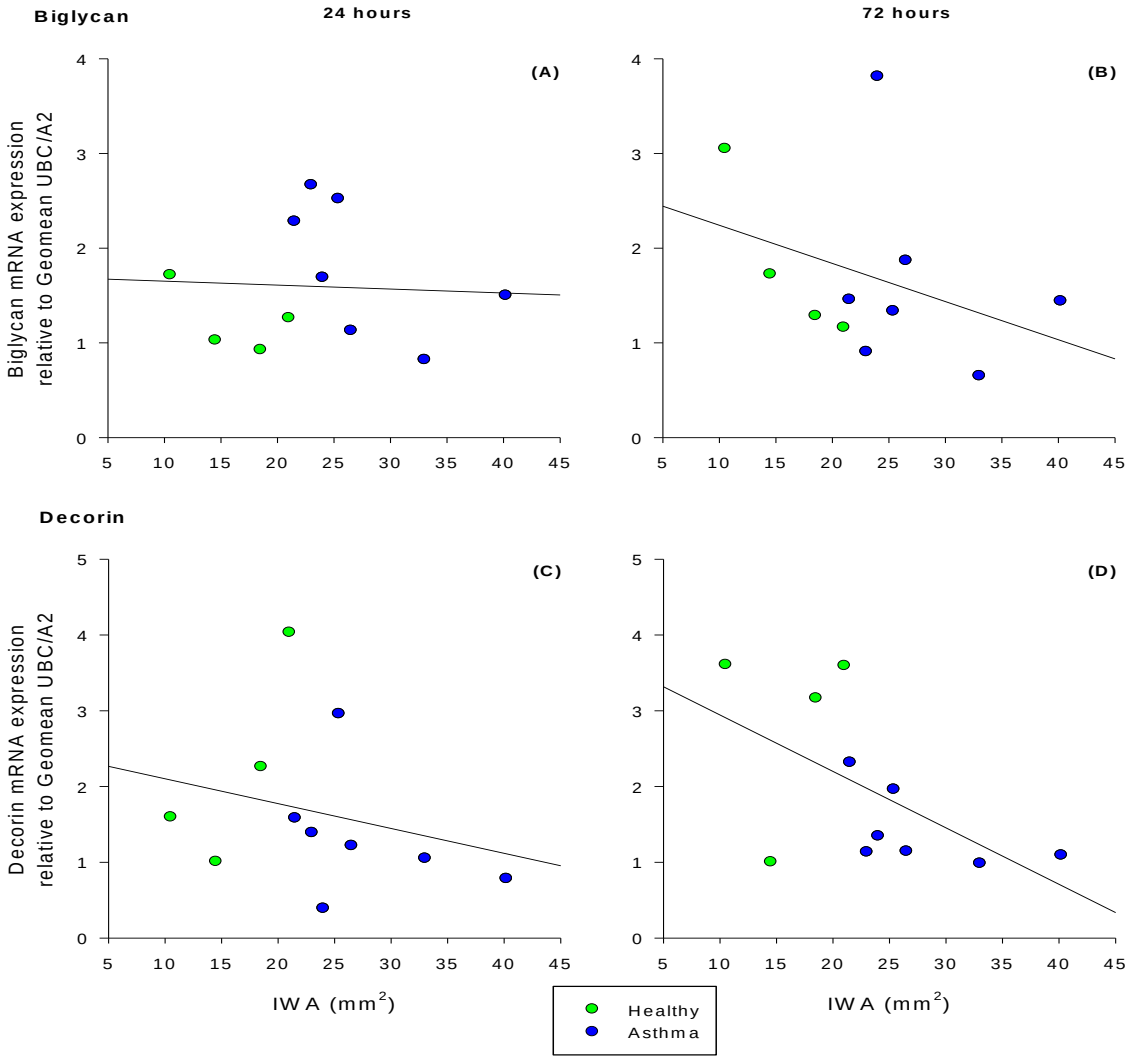

Figure 5.6. Correlation of Biglycan and Decorin proteoglycan mRNA expression by primary fibroblasts and inner airway wall area.

Biglycan mRNA expression at 24 hours (A) and 72 hours (B) and Decorin mRNA expression at 24 hours (C) and 72 hours (D) correlated to inner wall area (IWA).

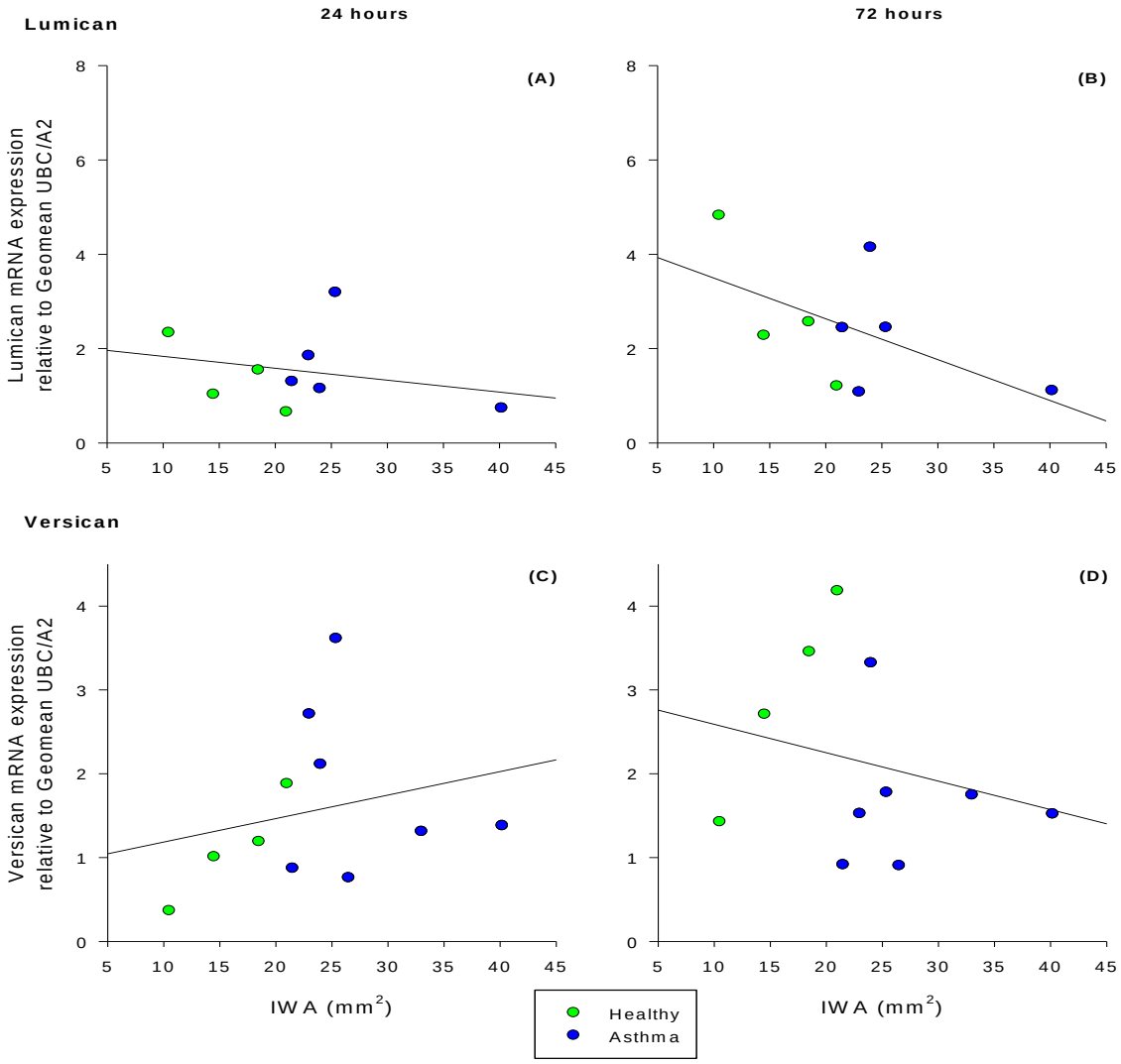

Figure 5.7. Correlation of Lumican and Versican proteoglycan mRNA expression by primary fibroblasts and inner airway wall area.
Lumican mRNA expression at 24 hours (A) and 72 hours (B) and Versican mRNA expression at 24 hours (C) and 72 hours (D) correlated to inner wall area (IWA).

5.4.2 Stimulated fibroblast proteoglycan mRNA

5.4.2.1 Stimulation with TNFα

Exposure of the cell cultures to TNFα did not seem to have any significant effect on the expression of these proteoglycans at the mRNA level neither at 4 hours nor at 24 hours. Only lumican shows a slight tendency to up-regulation with the TNFα (Figure 5.8 and Figure 5.9). The 72 hour measurements (not shown) were no different from the 24 hour ones.

5.4.2.2 Stimulation with $TGF\beta_2$

On stimulation with $TGF\beta_2$, some trends were discernible at 24 hours, although there were no changes at earlier time-points (data not shown). Decorin mRNA (Figure 5.11) appears to be down-regulated, whilst biglycan (Figure 5.10) and versican (Figure 5.13) are up-regulated. $TGF\beta_2$ does not appear to have any obvious effect on lumican output (Figure 5.12).

Protein expression, as determined via ELISA, for decorin (Figure 5.14) and versican (Figure 5.15) parallels the mRNA pattern. As would be expected, in contrast to the pre-translation elements the proteins show a cumulative upregulation (for versican) and downregulation (for decorin) over the 3 days.

Both the asthmatic and non-asthmatic fibroblast cultures appear to be behaving in a similar way with regards to proteoglycan synthesis at baseline and in response to TNFα and $TGF\beta_2$ stimulation.

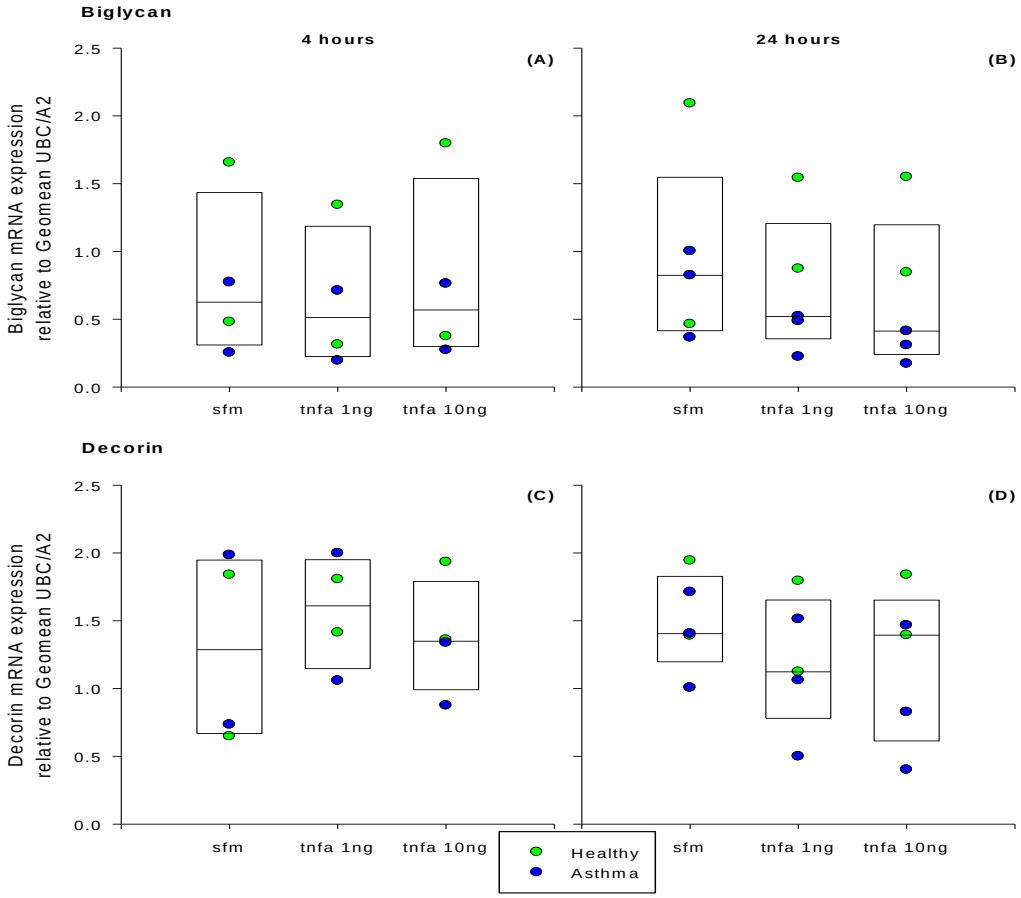

Figure 5.8. Expression of Biglycan and Decorin mRNA by lung primary fibroblasts in response to TNFα.

Biglycan mRNA output at 4 hours (A) and 24 hours (B) and Decorin mRNA output at 4 hours (C) and 24 hours (D) in response to 1ng/ml and 10ng/ml TNFα.

All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression at 4 hours of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratios: between fibroblasts from asthmatic and non-asthmatic subjects = 1.014, between treated and untreated fibroblasts = 1.006.

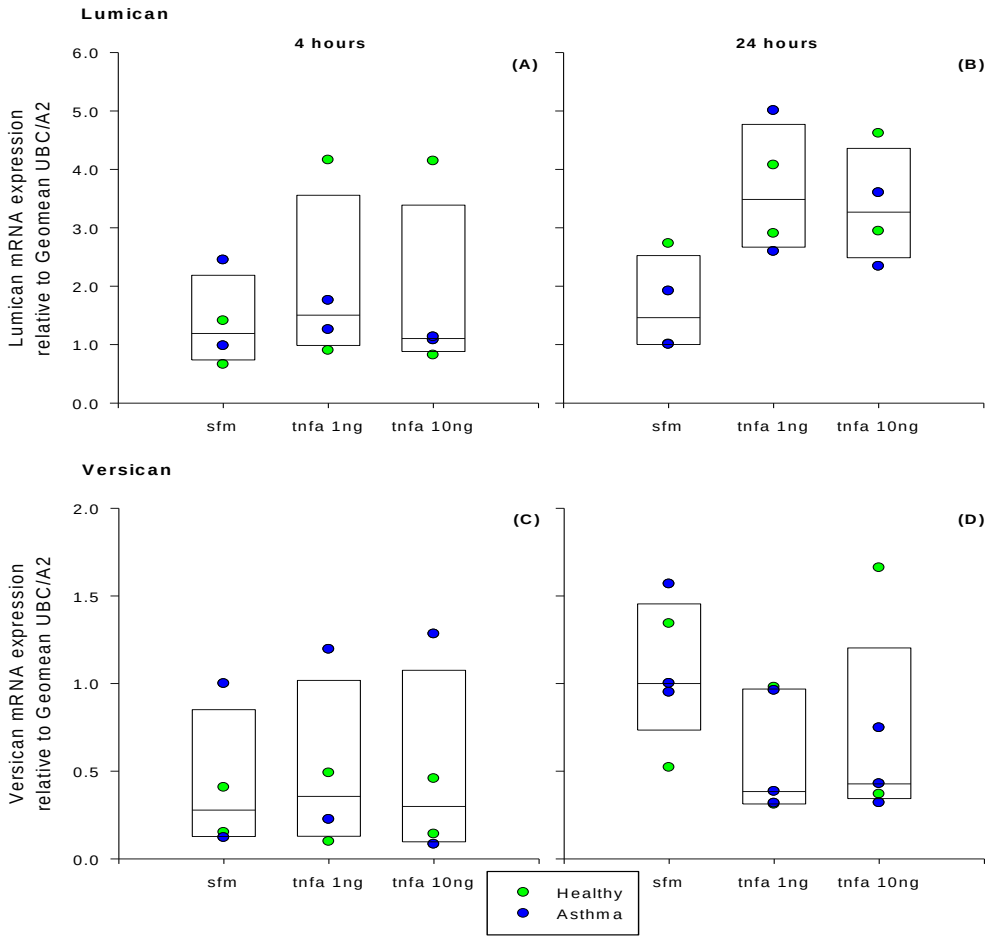

Figure 5.9. Expression of Lumican and Versican mRNA by lung primary fibroblasts in response to TNFα.

Lumican mRNA output at 4 hours (A) and 24 hours (B) and Versican mRNA output at 4 hours (C) and 24 hours (D) in response to 1ng/ml and 10ng/ml TNFα.

All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression at 4 hours of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratios: between fibroblasts from asthmatic and non-asthmatic subjects = 1.014, between treated and untreated fibroblasts = 1.006.

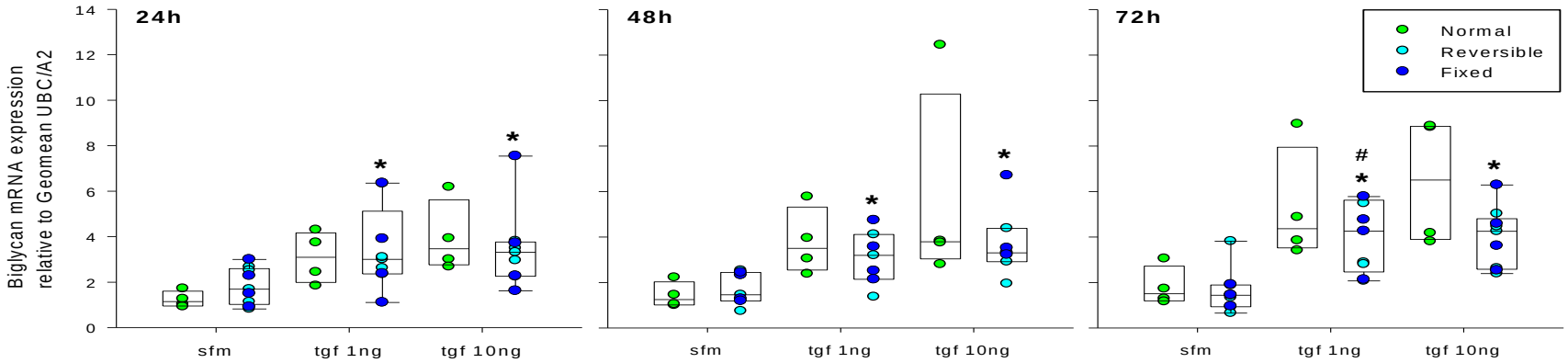

Figure 5.10. Expression of Biglycan mRNA by primary lung fibroblasts in response to TGFβ$_2$.

Data is shown for fibroblast cultures from healthy (n=4) and asthmatic (n=9) subjects. All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression at 24 hours of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratios: between fibroblasts from asthmatic and non-asthmatic subjects = 1.008, between treated and untreated fibroblasts = 1.005.

* = p <0.05 when compared to untreated sample at respective time-point.

= p <0.05 when compared to same treatment at 48 hours.

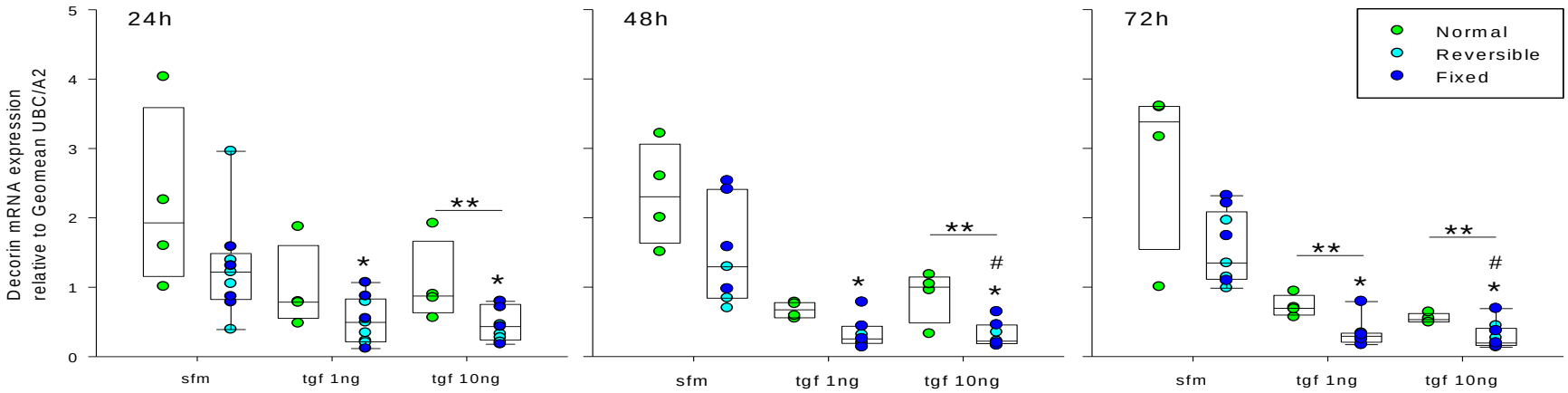

Figure 5.11. Expression of Decorin mRNA by primary lung fibroblasts in response to TGFβ$_2$.

Data is shown for fibroblast cultures from healthy (n=4) and asthmatic (n=9) subjects. All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression at 24 hours of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratios: between fibroblasts from asthmatic and non-asthmatic subjects = 1.008, between treated and untreated fibroblasts = 1.005.

* = p <0.05 when compared to untreated sample at respective time-point.

= p <0.05 when compared to same treatment at 24 hours.

** = p <0.05 when comparing asthmatic and non-asthmatic culture

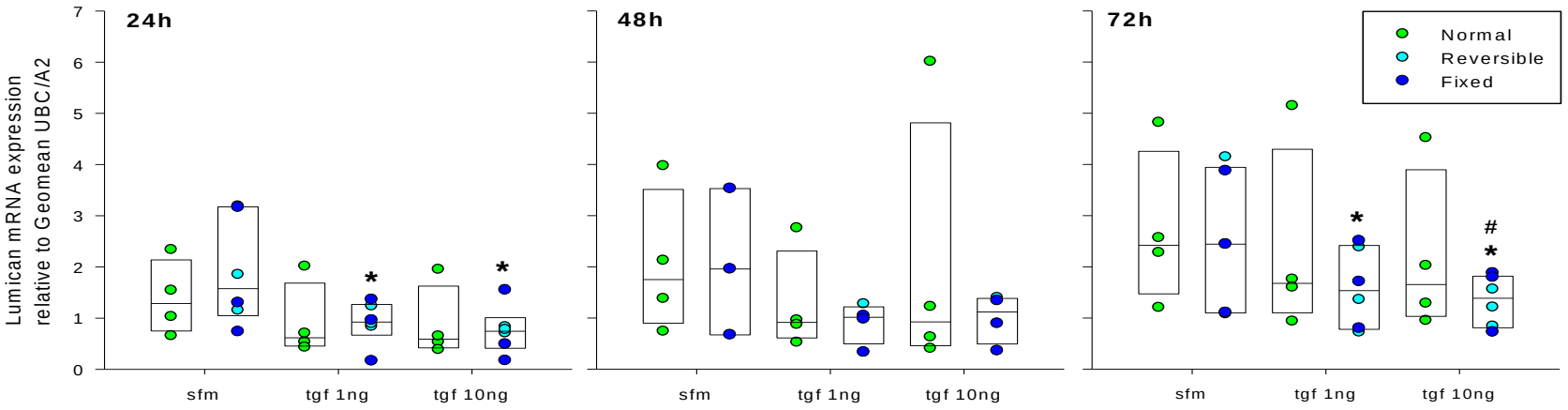

Figure 5.12. Expression of Lumican mRNA by primary lung fibroblasts in response to TGFβ$_2$.

Data is shown for fibroblast cultures from healthy (n=4) and asthmatic (n=6) subjects. All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression at 24 hours of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratios: between fibroblasts from asthmatic and non-asthmatic subjects = 1.008, between treated and untreated fibroblasts = 1.005.

* = p <0.05 when compared to untreated sample at respective time-point.

= p <0.05 when compared to same treatment at 24 hours.

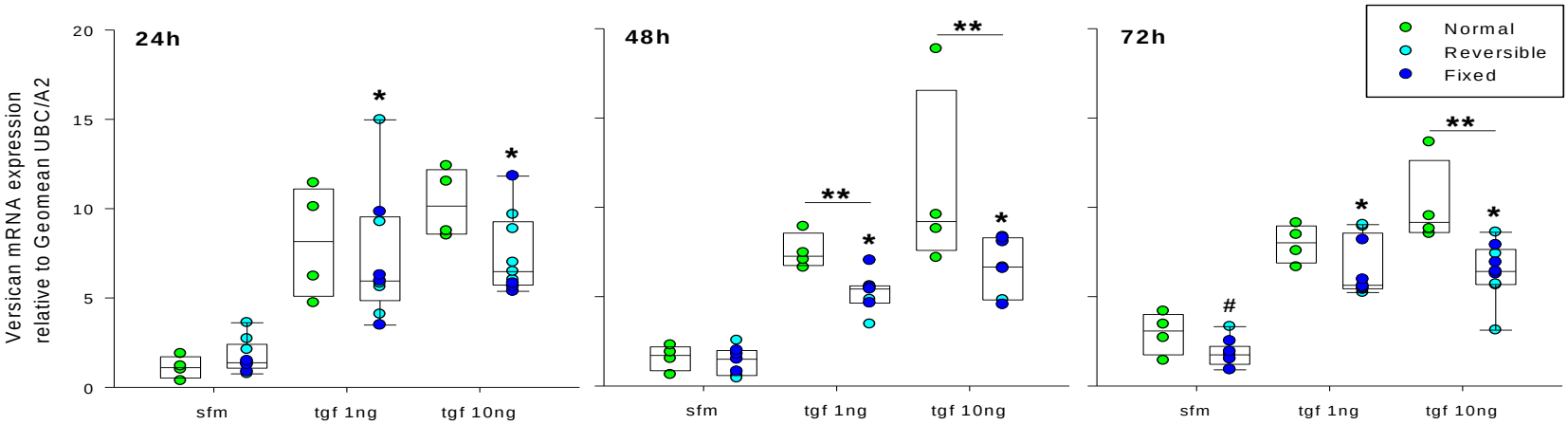

Figure 5.13. Expression of Versican mRNA by primary lung fibroblasts in response to TGFβ$_2$.

Data is shown for fibroblast cultures from healthy (n=4) and asthmatic (n=9) subjects. All values are normalised to reference genes A2 and UBC and expressed relative to the level of expression at 24 hours of one of the non-asthmatic fibroblast cultures.

Minimum detectable difference in expression ratios: between fibroblasts from asthmatic and non-asthmatic subjects = 1.008, between treated and untreated fibroblasts = 1.005.

* = p <0.05 when compared to untreated sample at respective time-point.

= p <0.05 when compared to same treatment at 48 hours.

** = p <0.05 when comparing asthmatic and non-asthmatic cultures.

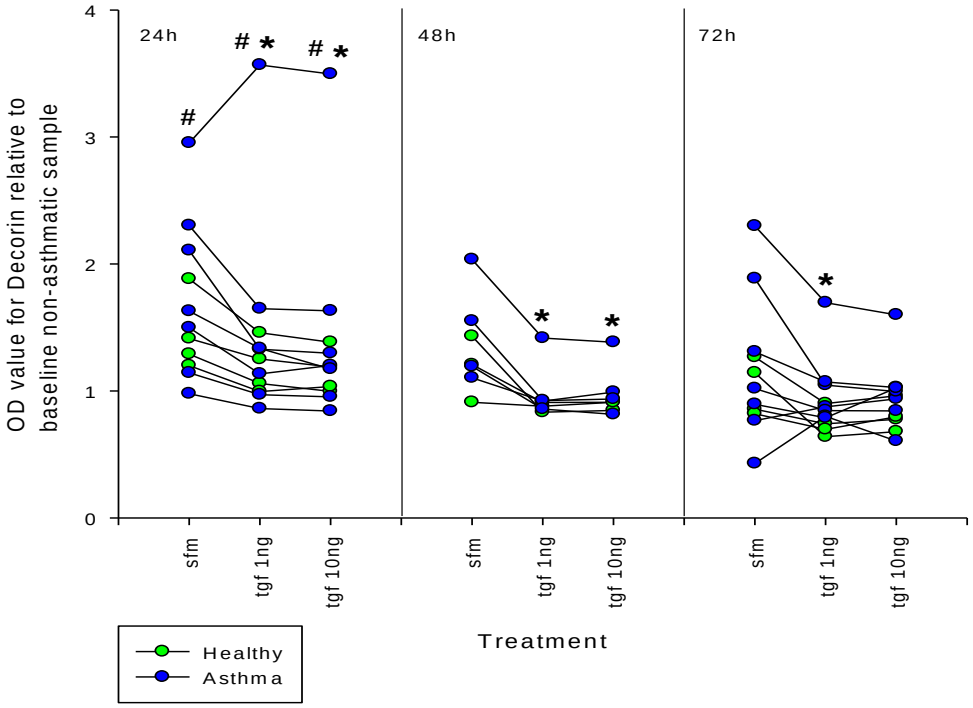

Figure 5.14. Production of Decorin protein by primary lung fibroblasts as determined by ELISA.

Data are shown for healthy (n=4) and asthmatic (n=7) fibroblast cultures. Values have been normalised to an unstimulated non-asthmatic culture at 24 hours.

* = p <0.05 when compared to untreated sample at respective time-point.

= p <0.05 when compared to same treatment at 48 and 72 hours.

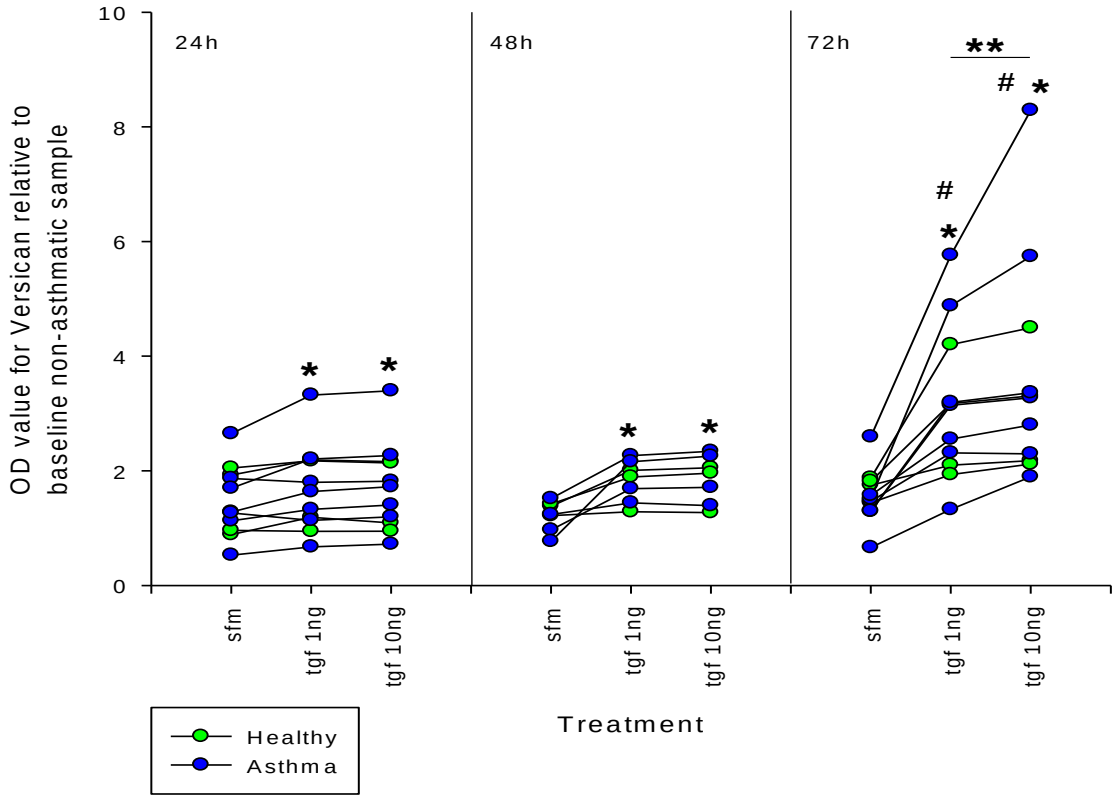

Figure 5.15. Production of Versican protein by primary lung fibroblasts as determined by ELISA.

Data are shown for healthy (n=4) and asthmatic (n=7) fibroblast cultures. Values have been normalised to an unstimulated non-asthmatic culture at 24 hours.

* = p <0.05 when compared to untreated sample at respective time-point.

= p <0.05 when compared to same treatment at 24 and 48 hours.

5.5 Discussion

The aim of this work was to determine differences in extracellular matrix protein synthetic profiles of primary fibroblasts. No differences could be identified in the baseline unstimulated proteoglycan synthesis between non-asthmatic and asthmatic fibroblasts from subjects with or without fixed airway obstruction. There was no correlation between the inner wall area as measured by endobronchial ultrasound and the fibroblast production of mRNA for the four proteoglycans under investigation.

Stimulation with TNFα did not seem to have any effect on the output of these proteins by the fibroblasts. This contrasts to some degree with the literature where a study [399] using embryonic lung fibroblasts has shown that TNFα induces about a 1.5-fold increase in biglycan and a 0.6-fold decrease in decorin at both the mRNA levels at 48 hours and the protein levels at 96 hours. The data presented here were measured at 24 and 72 hours. The same study reports no effect on versican levels. No data regarding lumican and TNFα could be found in the literature.

On the other hand, TGFβ appears to actively modulate the expression of these extracellular matrix proteins. It appears to upregulate versican and biglycan and down-regulates decorin. Similar findings have been reported in human skin and gingival fibroblasts in response to $TGF\beta_1$ stimulation [400]. The same findings have also been shown, with respect to biglycan and versican, in bleomycin-exposed rat lung fibroblasts treated with $TGF\beta_1$ [401] and in airway fibroblasts from COPD patients and healthy volunteers with respect to versican [402]. Most of these studies have demonstrated parallel changes in the protein expression.

Immunostaining for collagen 1 and 3 in central airway endobronchial biopsies showed increased expression in the asthmatic samples. cDNA from the same

primary fibroblasts used for the proteoglycan work was also analysed for expression of collagen 1 and 3 mRNA (data not shown). There was no difference in mRNA output between non-asthmatics (n= 4) and asthmatics (n= 7). No correlation with inner wall area was evident for collagen 1. Collagen 3 showed a significant negative correlation at 72 hours. The mRNA trends do not follow the same pattern as the protein levels. Indeed, surprisingly, there appears to be evidence of a negative correlation. So although the thicker airways have more collagen 1 and 3 within them, fibroblasts from this same category do not produce more collagen. Collagen in the airway is produced principally, but not exclusively by fibroblasts – other mesenchymal cells such as smooth muscle cells may be accounting for the difference in the amount of final product. The presence, concentration and level of activity of metalloproteinases in the airway wall would also be of relevance.

There are inherent limitations in studying fibroblasts in isolation. The fibroblast protein output for proteoglycans correlates with mRNA output for versican and decorin. However, as highlighted by the collagen data, this does not necessarily imply that the expression within the airway will follow suit.

Extracellular matrix remodelling is very likely to involve cross-talk between different cell types in the bronchial wall, particularly between epithelium and mesenchymal cells. No single mediator of epithelial origin has emerged as a principal driver of fibroblast or smooth muscle activity. Such a factor would provide a potential therapeutic target.

CHAPTER 6

Galectin-3 as a Potential Epithelial Pro-fibrogenic Factor

6.1 Introduction

A lot of research into asthma remodelling has been targeted at unravelling the complex interplay of inflammatory and bronchial wall cells that in certain instances results in remodelling. Over the past few years, more data have been generated on the role of protein-carbohydrate interactions and how they fit into this mosaic. Galectins have emerged as important modulators of the immune response with numerous postulated roles [403]. One of these, galectin-3 has been implicated in both the innate and adaptive immunity [404] as well as in fibrotic processes.

6.2 Galectins

Galectins are a family of lectins, multivalent proteins or glycoproteins which can form reversible complexes with mono- or oligosaccharide structures. Galectins preferentially bind to β-galactosides (molecules consisting of galactose bound to a non-sugar via a β-glycosidic bond) through a highly conserved carbohydrate-recognition domain. They are capable of multivalent binding either via the presence of multiple carbohydrate recognition domains on the same molecule or through dimerization. This enables higher affinity binding to ligands with multiple recognition sites or clusters of ligands, effectively resulting in binding to different carbohydrates or to different sites on the same carbohydrate. This multivalent binding through weak and reversible bonds is the principal way in which they mediate their numerous functions.

They are classified into 3 sub-classes depending on their architectural conformation – the proto-type class, the chimera class and the tandem-repeat class (Figure 6.1).

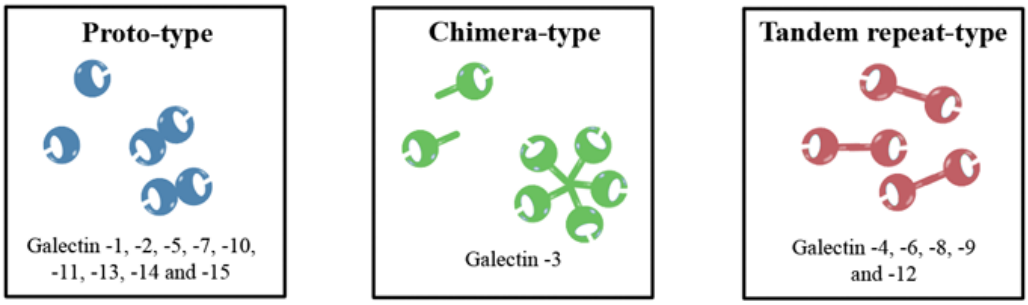

Figure 6.1. Schematic representation of the structure of members of the galectin family. Modified from [404].

They are all characterised by a carbohydrate-recognition domain (CRD) through which they can cross-link carbohydrates either by having more than one CRD on the same molecule or by forming multimers [405-408].

The functions attributed to galectins are many and varied. It is thought that a number of the functions are mediated by the formation of galectin-glycoprotein lattices at various sites. These lattices may be implicated in prevention of endocytosis of receptors, host-pathogen interactions or ligand clustering [409].

6.3 Galectin-3

About 15 mammalian galectins have been described to date. Galectin-3 is the sole member of the chimera group, so called because at one end it has the carbohydrate-recognition domain whilst at the other end there is an N-terminal domain with tandem repeats of proline- and glycine-rich sequences [410]. The latter has been implicated in the non-classical secretion of galectin-3 [411, 412] and in the formation of multimers - galectin-3 has been described as forming pentamers in vitro [413].

Galectin-3 has been identified in a number of tissues and cell types – including inflammatory cells particularly monocytes / macrophages [414], dendritic cells and epithelial cells [415, 416].

6.3.1 Functions of Galectin-3

In vitro experiments in a number of cell types have localised it to the nucleus [417], cytoplasm [418], cell membrane [419] as well as extracellularly as a secreted protein [420].

It has been shown to play a role in cell survival [421, 422], apoptosis [423, 424] and neoplastic transformation [425, 426], modulation of cell adhesion [427, 428] including interaction of neoplastic cells with the extracellular matrix [429] and cell proliferation [430].

Galectin-3 also appears to be involved in inflammatory processes [403] where it seems to be mostly pro-inflammatory. It has been implicated in B cell differentiation [431], T cell line growth [432], modulation of T and B cell apoptosis [418], adhesion of neutrophils to laminin [429] and endothelial cells [433], mast cell degranulation [434], promotion of monocyte / macrophage migration [435] and potentiation of phagocytosis [436]. It has been shown to promote respiratory burst in neutrophils [437], induce mediator release by mast cells and act as a chemoattractant for monocytes and macrophages [438]. As it has an overall anti-apoptotic effect, it may also be promoting the persistence of inflammatory cells.

Galectin-3 has also been implicated in fibrotic processes where it has been hypothesised to act either by inhibiting the endocytosis of TGF-beta receptors by cross-linking them, or by being involved in the TGFβ signalling pathway intracellularly [439]. It has been localised to areas of fibrosis in chronic pancreatitis [440] as well as in liver fibrosis. Galectin-3 has been shown to activate cardiac fibroblasts to produce collagen [441], whilst a galectin-3 knock-

out mouse model of liver injury failed to produce normal levels of procollagen 1 mRNA [439].

In vitro experiments with colonic epithelial cells have shown that galectin-3 produced by them is able to activate lamina propria fibroblasts [442]. This makes galectin-3 an interesting candidate as a link between epithelium and subepithelial fibroblasts.

6.3.2 Galectin-3 in Asthma

Higher levels of galectin-3 have been measured in the bronchoalveolar lavage fluid of subjects with idiopathic pulmonary fibrosis and interstitial pneumonia associated with collagen vascular disease [443]. Immunostaining for galectin-3 in chronic obstructive pulmonary disease (COPD) showed increased staining in the small airways epithelium of COPD subjects compared to healthy non-smokers and smokers [444].

There are no human studies that have looked at galectin-3 in asthma. However, in animal studies using a murine model of allergic airway inflammation, ovalbumin-sensitised and challenged mice showed higher levels of galectin-3 in lavage fluid than saline-challenged mice. When galectin-3 knock-out mice were subjected to the same treatment, they had less eosinophil recruitment, less goblet cell metaplasia and lower airway hyperresponsiveness when compared to the wild-type variety [445].

6.4 Aims

Galectin-3 may therefore have a role in human asthma. Increased production by asthmatic epithelium may be a stimulus in the switching of fibroblasts to myofibroblasts and hence in promoting a pro-fibrotic micro-environment.

The hypothesis being investigated here is that there are higher levels of galectin-3 in asthmatic epithelium than in non-asthmatic. Furthermore, on the basis of the findings in the animal model, the possibility that allergen challenge increases galectin-3 is also investigated.

6.5 Materials and Methods

6.5.1 Subject characteristics

Asthmatic and healthy volunteers were recruited. They were classified into mild and severe asthmatics using GINA classification – intermittent, mild and moderate persistent were considered as mild and severe persistent as severe asthma. At bronchoscopy brushings, biopsies and bronchoalveolar lavage were obtained as described in Chapter 2.

Subject characteristics varied for the different sample types. They are outlined in Table 6-1 to Table 6-4.

Table 6-1. Subject characteristics (n=17) for bronchial brushings.

	Healthy	Mild asthma	Severe asthma
Disease State	6	2	9
Age* (yrs)	41.67 (19-64)	43.50 (20-67)	46.89 (17-63)
Gender F : M	1 : 5	2 : 0	6 : 3
FEV_1 pre-BD* (% predicted)	105.7 (89-133)	103.3 (101-106)	63.7 (29.8-118.6)
Atopic	3	2	5
ICS* (mcg/day BDP equivalent)	0	425 (50-800)	2307 (1000-4000)
No. on OCS	0	0	1
Asthma duration* (yrs)	0	14 (10-18)	19.6 (1-41)

Table 6-2. Subject characteristics (n=30) for bronchial biopsies.

	Healthy	Mild asthma	Severe asthma
Disease State	7	15	8
Age* (yrs)	37.9 (19-64)	34.9 (19-51)	38.6 (17-59)
Gender F : M	3 : 4	7 : 8	7 : 1
FEV_1 pre-BD* (% predicted)	103.2 (89.8-125)	88.9 (74.2-106)	62.6 (35.6-86.7)
Atopic	2	9	4
ICS* (mcg/day BDP equivalent)	0	733 (400-1000) 12 steroid-naïve	2757 (500-4000)
No. on OCS	0	0	3
Asthma duration* (yrs)	0	19 (1.5-44)	18.6 (5-28)

Table 6-3. Subject characteristics (n=88) for bronchoalveolar lavage.

	Healthy	Mild asthma	Severe asthma
Disease State	25	41	22
Age* (yrs)	30.4 (19-54)	30.4 (18-50)	35.2 (18-67)
Gender F : M	17 : 8	17 : 24	18 : 4
FEV_1 pre-BD* (% predicted)	102.8 (88.2-123)	88.3 (64.6-112)	62.4 (30.7-98.4)
Atopic	3	30	12
ICS* (mcg/day BDP equivalent)	0	0	3247 (1600-8000)
No. on OCS	0	0	9

Table 6-4. Subject characteristics (n=11) for allergen challenge.

	Healthy	Mild asthma	Severe asthma
Disease state	0	11	0
Age* (yrs)		26.2 (23-41)	
Gender F : M		8 : 3	
FEV_1 pre-BD* (% predicted)		96.2 (85.2-114.7)	
Atopic		11	
ICS		0	

*Data shown as mean with range in brackets.
FEV_1 pre-BD = forced expiratory volume in 1 second as percentage predicted prior to bronchodilation.
ICS = inhaled corticosteroid, BDP = beclometasone dipropionate.
OCS = oral corticosteroid.

6.5.2 Allergen challenges

Allergen challenges were performed on 11 house dust mite sensitised mild asthmatics using freeze dried house dust mite (Dermatophagoides pteronyssinus) allergen extract (Diagenics Ltd, Milton Keynes, UK) made up with 0.9% saline to give a working concentration of 5000 U/ml. A baseline nebulised 0.9% saline dose was followed by incremental doses of allergen – 5, 10, 40, 160, 720, 2100 and 5000 U – delivered via a breath activated dosimeter connected to a controlling computer (APS pro nebuliser and controller software (JLAB v. 5.02.0) Viasys Healthcare, Germany). FEV_1 was recorded at 5, 10 and 15 minutes after each dose. The challenge was terminated if there was a fall in FEV_1 of ≥15% or maximum dose was reached. Post-challenge FEV_1 was measured at 20, 30, 45 and 60 minutes and then at 30 minute intervals up to 10 hours after the final allergen dose. Three challenges were performed at 48 hour intervals.

Bronchoscopy with lavage was performed before challenge (minimum of 14 days before first challenge) and then repeated 4 days after last challenge.

6.5.3 Brushings – qPCR

Bronchial wall brushings were taken at bronchoscopy as described in Section 2.3.2. The brushings were transferred to Trizol™ and RNA extracted and reverse transcribed using MMLV RT enzyme (Sections 2.8 and 2.9).

Levels of galectin-3 mRNA were measured using a primer/probe for LGALS3 (PrimerDesign, UK) via real-time qPCR (iCycler, BioRad). Values were normalised to reference genes UBC and (glyceraldehyde 3-phosphate dehydrogenase) GAPDH, chosen on the basis of work previously performed in our labs.

6.5.4 Biopsies – immunohistochemistry

Bronchial biopsies were obtained from segmental carinae using disposable biopsy forceps (Section 2.3.3) and embedded in glycol methacrylate as described in Section 2.5.1. Two micron sections were then cut, stained with a monoclonal mouse antibody to human galectin-3 (Abcam, Cambridge, UK) diluted at 1:100. Staining was then detected using a peroxidase-streptavidin system. Staining quantification was performed with the help of computer-assisted image analysis (Zeiss KS 400 image analysis system, Welyn Garden City, UK).

6.5.5 Bronchoalveolar lavage – ELISA

Bronchoalveolar lavage fluid was retrieved at bronchoscopy by wedging the scope in a segmental bronchus of the right upper lobe and instilling 120 ml of saline. Fluid was then suctioned out and collected. Cells were spun out and the supernatant stored at -80°C.

Enzyme-linked immunosorbent assay was performed on the lavage supernatant using a galectin-3 ELISA kit (Calbiochem, Nottingham, UK).

6.5.6 Statistics

Non-parametric statistical analysis (Kruskal Wallis, Mann-Whitney U, Wilcoxon Signed Ranks test) was used to compare differences between the groups. A p value of <0.05 was taken to denote statistical significance.

6.6 Results

6.6.1 qPCR

Epithelial galectin-3 mRNA expression was assessed in bronchial brushings. Galectin-3 mRNA was expressed by the epithelium but there was no difference in levels between the asthmatics and non-asthmatics (Figure 6.2).

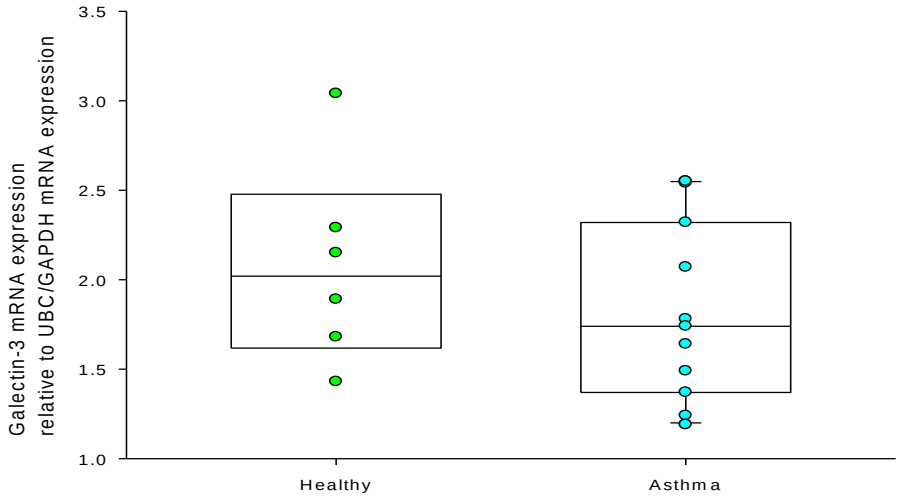

Figure 6.2. Galectin-3 mRNA expression in bronchial brushings.
Analysis performed in asthmatic (n=11) and non-asthmatic (n=6) brushings normalised to the geometric mean of UBC and GAPDH mRNA and expressed relative to the average ΔCt of the healthy samples. Mann-Whitney U test p = 0.404.

6.6.2 Immunohistochemistry

In order to quantify the presence of the protein within the bronchial wall, immunohistochemistry was used. Staining for galectin-3 was isolated to the bronchial epithelium, as reported in the COPD study [444]. As can be seen in Figure 6.3, the staining was mostly within the cytoplasm of the ciliated columnar epithelial cells, seeming to spare basal epithelial cells, with some protein localised to the brush border.

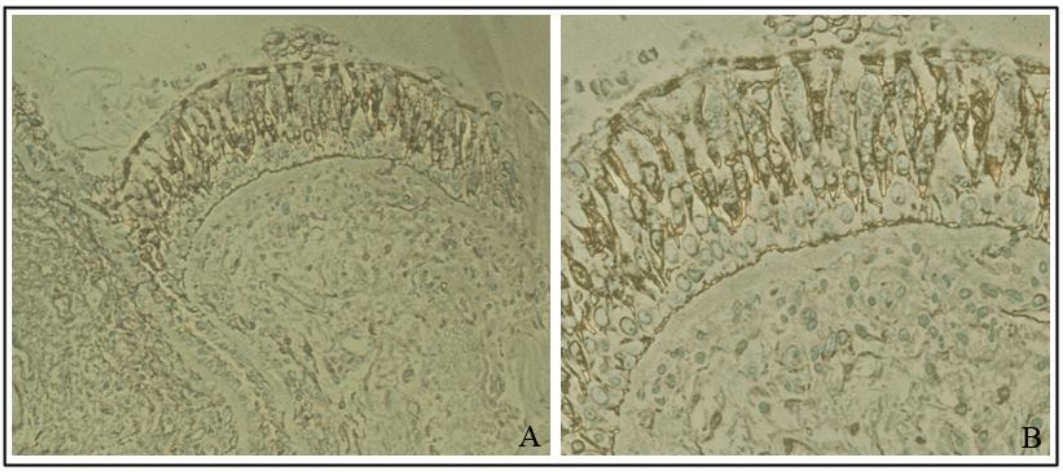

Figure 6.3. Representative images of Galectin-3 protein expression and localisation in bronchial wall biopsies by immunohistochemistry.
Both photomicrographs are from an asthmatic biopsy, at x10 magnification (A) and x20 magnification (B). Biopsies stained with isotype matched IgG control were completely clear.

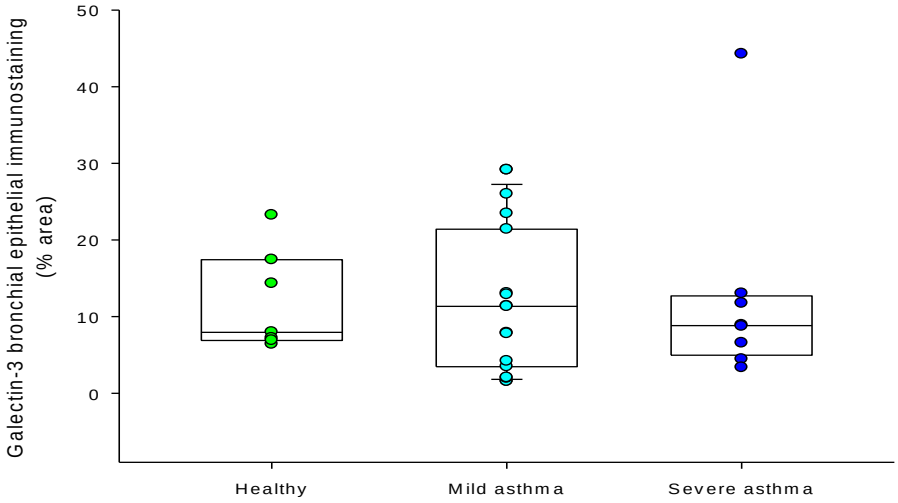

Figure 6.4. Galectin-3 percentage area staining in bronchial epithelium. Bronchial biopsies from healthy subjects (n=7), mild asthmatics (n=15) and severe asthmatics (n=8) were measured. Kruskal Wallis p = 0.934.

Thirty biopsies with a preserved epithelium of at least 0.8 mm length were analysed for percentage area staining using a computer software application. No significant differences in the presence of the protein in the epithelium were identified (Figure 6.4).

6.6.3 ELISA

As galectin-3 is known to be a secreted protein, ELISA for galectin-3 was performed on bronchoalveolar lavage fluid from 88 subjects. The three groups had very similar levels – 7.85 (1 – 22.3) ng/ml in healthy volunteers, 8.02 (1.9 – 24.5) ng/ml in mild asthmatics and 8.19 (0.29 – 26.4) ng/ml in severe asthmatics. The differences did not reach statistical significance (Figure 6.5).

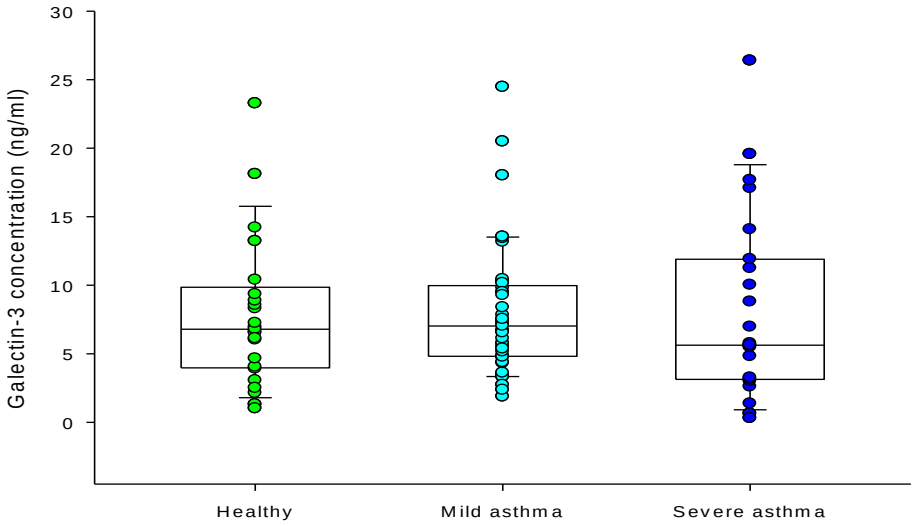

Figure 6.5. Galectin-3 concentration in bronchoalveolar lavage fluid.
ELISA was performed on bronchoalveolar lavage fluid from healthy volunteers (n=25), mild asthmatics (n=41) and severe asthmatics (n=22). Kruskal Wallis p = 0.904.

In view of the findings in the experiments on the murine asthma model [445], BALF galectin-3 was also measured in a group of asthmatics pre- and post-allergen challenge. This showed an increase in galectin-3 following challenge (Figure 6.6).

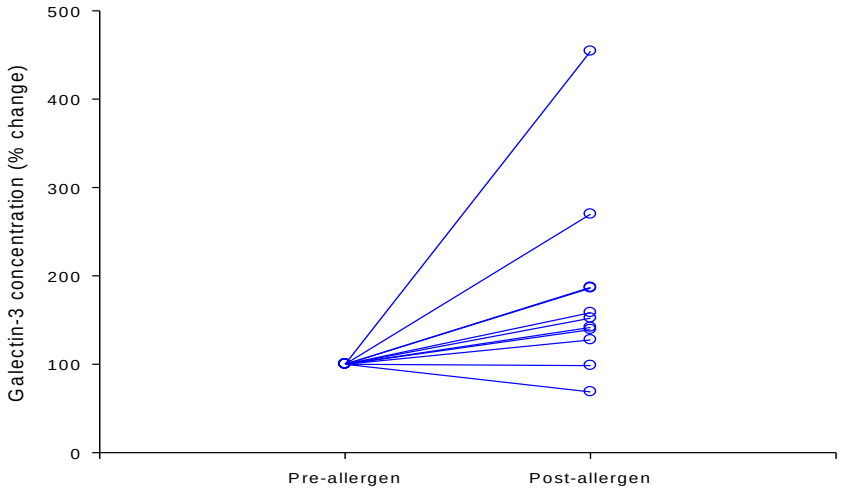

Figure 6.6. Percentage change in galectin-3 concentration in bronchoalveolar lavage fluid before and after allergen challenge. Wilcoxon signed ranks test for absolute concentrations of galectin-3 in BALF p = 0.016.

6.7 Discussion

In the experiments outlined, the presence of galectin-3 has been demonstrated in the epithelium of large airways as well as in the bronchoalveolar lavage fluid of asthmatics and healthy volunteers. Despite the suggestive in vitro and animal study data, no significant difference in the levels of galectin-3 at either the RNA or protein levels could be demonstrated.

These experiments essentially give an overall view of galectin-3 production in the bronchial wall. Important differences in the micro-localisation of this protein may exist and could be playing relevant roles in the pathogenesis of asthma.

In contrast to the data presented here, this protein has been shown to be relevant in other lung diseases such as pulmonary fibrosis and COPD. There could be a number of reasons for this. There are basic differences in the cytokine profile of these diseases when compared to asthma. It is also important to note certain differences in the study set-ups which may have had an impact on the difference in results.

The COPD study [444] found significant differences in immunostaining in the small airways in lobectomy samples. The 'healthy' smokers and non-smokers were undergoing lung surgery for a solitary pulmonary nodule. Our samples were retrieved at bronchoscopy and therefore were obtained from more central airways.

In the pulmonary fibrosis study [443], which is the only study we are aware of where galectin-3 levels were measured in human bronchoalveolar lavage, the levels measured in healthy volunteers were lower than what we found. They had a mean value of 3.19 ± 1.6 ng/ml for their 6 healthy volunteers, whilst the mean value for our 25 non-asthmatic controls was 7.85 ng/ml with a range of values between 1 and 22.3 ng/ml. The smaller numbers in that study could have resulted in a type I error in the interpretation of data.

In the murine model of asthma using galectin-3 knock-out mice, a role for galectin-3 seemed very likely. There may be important differences between an acute allergen scenario, as was the case in the mouse model, and the more stable asthma we have in our subjects. In fact when galectin-3 BALF levels were measured in a group of mild asthmatics who had a bronchoscopy pre- and post-allergen challenge, there was a significant rise in levels. There is also the possibility that not all extrapolations from the animal model to the human disease are possible.

Another potential confounding factor is treatment. In the previously mentioned pulmonary fibrosis study [443], a non-significant tendency for lower levels of

galectin-3 was reported in steroid-treated collagen vascular disease associated interstitial pneumonia.

The only other study looking at effects of glucocorticoids on galectin-3 expression involves rat mammary epithelial cells undergoing involution post-lactation. Involution seems to involve upregulation of galectin-3 which is suppressed if the animal is treated with dexamethasone. Interestingly, although there was a lower protein level, dexamethasone seems to have had no effect on the mRNA levels suggesting that it modulates post-translational processes or affects protein stability [446].

All the severe asthmatics in our study were on inhaled corticosteroids, whilst there was a mixture of cases in the mild asthmatics. As can be seen from Table 6-3, all mild asthmatics in the BALF cohort were steroid-naïve, whilst only 3 out of 15 in the bronchial biopsy group were on inhaled steroids (Table 6-2). The two mild asthmatics in whom mRNA was measured were both on inhaled steroids (Table 6-1).

Considering the relatively few subjects on steroids and the reported steroid effect on mRNA, it is probably safe to assume that these results are not confounded by steroid effect.

Protein-carbohydrate interactions offer another attractive avenue for research with regards to the cell-cell and cell-matrix interactions occurring in asthma. The galectin family, as can be inferred from the evolutionary conservation of the structure of its members, covers fundamental roles in cell biology. This gives the galectins involvement in diverse cell functions including inflammatory and fibrotic processes.

Galectin-3 appears to have a role in fibrotic processes in a number of organs including the lung. The data presented here would seem to suggest that acute and

chronic inflammation have different pathophysiology and galectin-3's role may be more important in the early post-allergen exposure phase of atopic asthma.

CHAPTER 7

Conclusions and Future Work

This work has evaluated airway remodelling in a number of ways. Airway wall thickness, as assessed by computed tomography, was compared with collagen immunostaining in endobronchial biopsies. Endobronchial ultrasound was used to assess airway wall dimension changes in healthy volunteers and asthmatics with a range of asthma severity. Proteoglycan output by fibroblasts was investigated in vitro, using primary fibroblast cultures from healthy subjects and asthmatics. And finally, the airway expression of galectin-3 was measured in a comparative study of healthy controls and asthmatics at baseline and additionally in asthma pre- and post-inhalation allergen challenge.

7.1 Collagens

7.1.1 Summary of findings

Collagen 1 and collagen 3 were found to show increased immunostaining in endobronchial biopsies from asthmatic subjects when compared to healthy controls.

There was a significant positive correlation between the degree of immunostaining of collagen 1 and collagen 3 and HRCT measures of airway wall thickness for the subjects studied.

7.1.2 Extracellular matrix components and airway wall thickness

Collagen 1 and 3 are well documented as components of the extracellular matrix that are increased in asthma [30, 129, 195, 447]. Asthmatics have also been shown to have increased airway wall thickness.

The data presented here confirm an increase in collagen in the airway walls of asthmatics as well as an increase in wall thickness. Correlating the two shows a

significant trend between amount of both collagen 1 and collagen 3 in the airway and measures of wall thickness. However, when analysing the asthmatic airways data in isolation the correlation loses its significance. This may suggest either lack of power in subject numbers or that the whole group correlation is merely reflecting the difference between the asthmatic and non-asthmatic subsets rather than a real link between amount of collagen in the wall and its overall thickness. One of the limitations of endobronchial biopsy analysis is that it is a single sample from a large airway wall and does not contain all components of the airway wall. Potentially larger number studies that allow for sample and disease variability might permit better evaluation of the relevance of interstitial airway wall collagen changes to airway wall dimensions.

Both extracellular matrix production and airway wall thickness are reflecting effects of remodelling processes. But apart from this, could there be a link between the two? Although never looked at specifically, the thickening of the airway in asthma could be attributable to a number of factors- oedema, thickening of the reticular basement membrane; but probably increased extracellular matrix and smooth muscle hyperplasia and hypertrophy are the major contributors.

ECM is not only produced by fibroblasts and myofibroblasts but also by airway smooth muscle cells [132, 448]. Components of the ECM in turn promote ASM survival [111] and proliferation [449, 450] as well as myofibroblast differentiation [175]. The net effect of this is an increase in ASM mass and a positive feedback loop increasing ECM production.

ECM and ASM mass can definitely affect airflow dynamics by altering airway mechanical properties and increased contractility respectively, as well as by increasing airway wall thickness. It is not just the total amount of ECM that is probably very relevant but also the sites within the wall where it is deposited.

Looking at the components of the airway wall and their relationship to extracellular matrix output could shed some further light on remodelling.

7.2 Endobronchial ultrasound

7.2.1 Summary of findings

This study shows that endobronchial ultrasound can be used safely in asthmatics including those with severe asthma. It adds, on average, about 5 to 10 minutes to the procedure and is well tolerated by the participants.

After a few initial adjustments to determine best site and orientation for inflation of balloon, there was good consistency in obtaining suitable images. The interpretation of the videos and images obtained requires a degree of repetition and review to achieve consistency but is achievable by a person with no training in ultrasonography.

The most significant advantage of endobronchial ultrasound is its ability to distinguish bronchial wall layers, which was possible in most of the participants. This gives access to information about the different components of the airway wall.

Using this imaging modality, it has been shown that the inner wall area of the bronchial wall is increased in severe asthma particularly where there is fixed airway obstruction. From the work done by Kurimoto *et al* [328], what is being referred to as the inner wall area corresponds to the submucosal and smooth muscle layers. These findings are in keeping with both HRCT data suggesting an increase in overall airway wall thickness in asthma and post-mortem studies showing an increase in thickness of individual components of the wall.

In this study, measurements of luminal area using endobronchial ultrasound are increased in severe asthma particularly where there is fixed airway obstruction. This was an unexpected finding which has not been reported elsewhere, although dilated airways are often reported in scans from severe asthmatics [214]. There was no convincing correlation with surrogate measures for airway trapping.

7.2.2 Endobronchial ultrasound as a research tool

These findings confirm increased airway wall thickness in severe asthma using another imaging modality. There is no single parameter or measure which defines airway remodelling but airway wall thickness probably provides a good end-point measure. Endobronchial ultrasound could provide another important tool with which to study remodelling.

The limitations of endobronchial ultrasound have to be borne in mind. There is an element of subjectivity in endobronchial ultrasound image interpretation which is probably relevant to all sonographic investigations. In this type of study, the precise cut off boundaries between layers is open to interpretation although reproducibility for an individual operator is probably achievable. For the purposes of research, this problem could be partially overcome by comparing the measurements obtained by two or more independent investigators. In the long term however, as image interpretation is always going to be subjective, it may be interesting to see if software, created for this specific purpose or modified from existing packages, could help in deriving reproducible measurements.

Furthermore, balloon inflation has an inherent potential to alter wall dimensions. There is scope for performing dedicated bronchoscopies to determine optimal volumes or pressures at which to inflate the balloon. This distortion potential could also be studied further on animal or human cadaveric bronchi by inflating the balloon to different degrees, although data from studies on sheep bronchi

suggest no difference when performing ex vivo measurements with a balloon interphase or without it [208].

Due to the fact that the procedure was performed endoscopically, the point in the breathing cycle at which the images were recorded could not be controlled precisely – which in turn may be a potential source of error. This factor would in practice be more complicated to eliminate as it would be quite difficult for subjects to control their breathing during a bronchoscopy. It may be possible to record a video over a few breathing cycles thereby allowing measurements at different points of inspiration and expiration, which could then be averaged.

Once consolidated as a reliable imaging modality, the biggest contribution for endobronchial ultrasound would be longitudinal studies to monitor and investigate the changes in airway wall thickness over time. Performing longitudinal repeat HRCT measures of wall dimensions would be inadvisable, in view of the heavy load of radiation incurred but would certainly be possible using endobronchial ultrasound. This could provide important information about the natural progression of wall thickening, and by inference airway remodelling, about which there is a paucity of information.

7.2.3 Endobronchial ultrasound as a clinical tool

Looking at these findings within the broader scope of clinical asthma, it is not unthinkable to envisage endobronchial ultrasound, with further optimisation, as an important tool in identifying subjects with more pronounced remodelling. This could in turn, determine the type of therapeutic intervention offered and provide a means of monitoring interventions. Assessing effects of therapies such as bronchial thermoplasty would certainly be possible.

Due to the use of non-harmful sound waves and ability to discriminate wall layers, it has distinct advantages when compared to computed tomography. It is however

an invasive procedure and therefore unlikely to find a role in the routine assessment of asthmatics but it certainly could be added on to a bronchoscopic examination in difficult-to-treat cases.

7.2.4 Increased luminal area – is it relevant?

Increased luminal area has never been reported in severe asthma, and indeed most HRCT measures quoted in other studies are ratios that incorporate in some way the luminal area. Increased luminal area is certainly counter-intuitive particularly in a group with a predominantly fixed airway obstruction.

Due to the need for a liquid interphase between probe and wall, necessitating the inflation of a balloon, there is the potential for airway distortion. All care was taken to minimise this both during the procedure and in the subsequent choosing of images, however it cannot be eliminated completely. Nevertheless, this should be a 'constant' error across all the subjects and would not explain the difference between subject groups.

The difference could be reflecting differing physical properties of the wall in more severe asthma, namely more easily distortable walls in severe asthmatics. The literature seems to suggest that airway walls in asthma are less, rather than more, compliant [351, 365, 451] although the actual studies are few and performed on relatively mild asthmatics.

The measurements made in this study refer to one specific site in the bronchial tree and do not necessarily reflect what is happening elsewhere. One possibility is that this group has a predominantly small airways disease with narrowing in the more distal airways. It is also conceivable that increased collapsibility rather than narrowing is responsible for the spirometric findings.

The number of subjects in each group is small and quite heterogeneous so comparisons may be confounded by varying physiological and treatment differences within each group. To some degree this is a more realistic picture of the spectrum of asthma encountered in the clinics and makes any identifiable differences even more relevant.

Severe asthma, particularly with fixed airway obstruction, has some similarities to COPD. Sorkness at al showed that severe asthma had prominent air trapping, evident as reduced FVC and RV/TLC over a range of FEV_1/FVC ratios [362]. The possibility that air trapping may, via traction, be the cause for the increased luminal area was investigated. Although there was a trend for the measured endobronchial area to follow measures of air trapping it did not reach statistical significance. This may be due to lack of power and increasing numbers could have clarified it. Obviously even a significant correlation would not prove causality and further work would be necessary.

Another imaging modality could be employed to look into luminal area within these subjects or ones with a similar phenotype to exclude the possibility that this is a technique error or a statistical fluke. Due to comfort and time constraints, the EBUS measurements were only performed at one point in a specific part of the bronchial tree and extrapolation to other parts of the airways is only hypothetical. Performing dedicated bronchoscopies, it would be possible to undertake measurements in other parts of the bronchial tree.

If increased luminal area is confirmed, it would be necessary to look further to elucidate the reasons behind it and determine its clinical significance, if any. Further investigation into lung volumes, in vivo and ex vivo assessment of airway wall mechanical properties and impact of pharmacological interventions could be looked into.

7.2.5 Fixed airway obstruction – does it identify an important subset of asthmatics?

There are only a handful of studies that have looked at fixed airway obstruction asthmatics. Work is probably hampered by the fact that there is no defined level of reversibility that distinguishes the reversible from the fixed.

A large study, looking at fixed airflow obstruction, compared baseline characteristics in 228 subjects at asthma diagnosis and after a follow-up period varying between 21 and 33 years. Fixed airway obstruction occurred in 16 percent and was associated with a lower baseline FEV_1, lower reversibility and, interestingly, less bronchial hyperresponsiveness [452]. This can be linked to the notion that airway remodelling protects against excessive airway narrowing. The endobronchial ultrasound data, although small in number, show a higher PC_{20} in the thicker airways. This was also shown, more robustly, using HRCT by Niimi *et al* [143].

Endobronchial ultrasound identified an increased inner wall area in asthmatics with a fixed airflow defect. This mirrors similar findings using CT scanning [214]. On the basis of this, it is safe to infer that this group of asthmatics have more pronounced remodelling. In the group presented here they were also using health resources significantly more than the 'reversible' group. 'Reversible' airways asthmatics showed a mean combined number of out-of-hours GP or A&E visits and hospitalisations of 2.54 per year whilst the 'fixed' airways asthmatics had 6.8 per year over the previous 12 months (Mann-Whitney U $p < 0.05$).

This group have slightly different symptomatology [452], different therapeutic needs and possibly distinct pathophysiology. They typically present later in life and tend to be less atopic [212] and probably constitute a genetically distinct group. Characterising larger numbers could help in determining spirometric

parameters which correlate with clinical symptoms. From an airway remodelling perspective, longitudinal studies looking at their airway walls at a macroscopic, cellular and molecular level would be very relevant.

7.3 Proteoglycans

7.3.1 Summary of findings

Proteoglycans are relatively large macromolecules which, together with collagens, are important constituents of the extracellular matrix. They can affect the mechanical properties of the airway, and may play roles in cell migration and proliferation both through modulation of the physical environment and via sequestration and compartmentalisation of cytokines and growth factors.

Airway fibroblast mRNA and protein output of various proteoglycans, namely decorin, biglycan, lumican and versican, was investigated in primary airway fibroblasts cultured in vitro. From previous studies, these seem to be the most relevant in asthma. No measurable difference was detected in proteoglycan generation by healthy and asthmatic airway fibroblasts. Neither was there any difference between the mild and severe asthma phenotypes.

When stimulated with $TGF\beta_2$, the fibroblasts showed a response with changes in the output of proteoglycans which could potentially be relevant in vivo. Biglycan and versican were upregulated whilst decorin and lumican were downregulated. Although never investigated specifically in lung fibroblasts, this is in keeping with findings in fibroblasts from other tissues. All changes were shown at an mRNA level for all four proteoglycans and paralleled in the actual protein output for decorin and versican. There was no difference in response between asthmatic and non-asthmatic fibroblasts.

Messenger RNA output of these proteoglycans showed no correlation with endobronchial ultrasound measures of airway wall thickness. Stimulation with TNFα and galectin-3 (data not shown) separately had no measurable effect on proteoglycan output.

7.3.2 Are proteoglycans important in remodelling?

There is a literature showing that there is an increased presence of proteoglycans in the asthmatic airway [130-132]. This work tried to determine whether this was attributable to increased synthesis by fibroblasts.

From these data, with respect to proteoglycans, asthmatic lung airway fibroblasts do not differ from non-asthmatic ones. Despite evidence for increased numbers of fibroblasts and myofibroblasts in the asthmatic airway, extensive study of these cells has failed to show any innate difference in the phenotype or synthetic profile of the asthmatic fibroblasts. This contrasts with airway smooth muscle cells where phenotypical differences are described between asthmatic and non-asthmatic airways, particularly in proliferation potential [112].

Interestingly, recent work on fibroblasts from COPD subjects [402] shows different proteoglycan production in peripheral fibroblasts when compared to healthy subject fibroblasts. Endobronchial biopsies were used to culture centrally-derived fibroblasts whilst explants and transbronchial biopsies were used to obtain peripheral fibroblasts. Peripheral fibroblasts from COPD patients had a 2-fold increased basal output of versican when compared to healthy controls. This was not the case for the central fibroblasts. On stimulation with $TGF\beta_1$, there was an increased output in versican and biglycan in fibroblasts from both sites, but no difference between those with or without COPD. However, distal fibroblasts from COPD showed increased response relative to central COPD ones. These data show that, in COPD, peripheral fibroblast populations may be more important. Although

there is no directly similar work in asthma, severe asthma particularly with a fixed airflow obstruction component does have some similarities to COPD. Furthermore, in 18 cases of fatal status asthmaticus, there was increased immunostaining for versican and decreased immunostaining for decorin and lumican in the small airways compared to controls which was not shown in the central airways [131].

Although the fibroblasts in more severe asthma are not phenotypically different and show a synthetic profile which is similar to that of mild asthmatics and non-asthmatic airway fibroblasts, they are potentially exposed to increased levels of TGFβ, particularly in severe asthma [141, 195, 396, 453-456]. This could influence their output of extracellular matrix proteins.

Since these data show that the increase in proteoglycans is likely to depend on the cytokine environment in which these cells find themselves in asthma rather than any innate differences in the fibroblasts themselves, it would certainly be worthwhile to look at other mediators apart from TGFβ or indeed combinations of cytokines. One attractive idea would be to stimulate them with BALF from asthmatics of varying severity as a surrogate of the intramural cytokine mix.

Mechanical strain has been shown to alter proteoglycan output by asthmatic fibroblasts [457, 458], so mechano-stimulation, apart from the cytokines surrounding the cells, would probably be very relevant within the airway.

Apart from the influence of cytokines and mechanical stimulation, the increased numbers of fibroblasts described in asthmatic airways, may also mean that despite no difference in synthesis by individual cells, the overall output would be higher.

These data are derived from fibroblasts cultured in vitro in artificial media without the normal influence of adjacent cells and extracellular components normally present within the bronchial wall or the impact of mechanical forces they are

normally subjected to in the airways. This obviously creates an artificial scenario which is necessarily a simplified version of reality.

Proteoglycans can be produced by airway smooth muscle cells [459] apart from fibroblasts and relevant contributions by these cells were not taken into account in this work. Co-culture of primary lung fibroblasts and airway smooth muscle cells could provide an overview of the relative output from these two cell types as well as take into account the effect of cross-talk between them.

The sum total of the amount of proteoglycans within the airway wall is determined by both synthesis and degradation both of which are plastic processes influenced by numerous factors. The fibroblast data presented here looks only at the synthesis of proteoglycans. Endobronchial biopsies were retrieved at bronchoscopy for all subjects on whom ultrasound airway thickness data are available. Immunohistochemistry to compare proteoglycan presence in airways of different thickness could be performed.

7.3.3 Are proteoglycans clinically significant?

Although the increase in airway wall proteoglycans in asthma is a well-established fact, the exact significance of this is not completely clear. There is, however, less information about the proteoglycans' impact on wall characteristics. From the perspective of this work, the main interest in proteoglycans lay in whether they may partly be responsible for fixed airflow obstruction.

The fibroblasts grown from endobronchial biopsies of the asthmatic subjects did not show a different proteoglycan message, whether the subject had increased or normal airway wall thickness (data not shown), or whether there was a fixed airflow defect. Naturally, proteoglycan accumulation could be resulting from increased fibroblast numbers, synthesis by non-fibroblast cells or diminished degradation. This would seem to suggest that the basal synthesis of

proteoglycans by central fibroblasts may not be an important contributor to airflow limitation or airway thickness.

Two studies have identified positive correlations between certain proteoglycans and airway hyperresponsiveness. One showed that subepithelial immunohistochemical staining for lumican, biglycan and versican in endobronchial biopsies from 7 mild asthmatics showed a negative correlation with their PC_{20} [130]. A similar correlation was described for the presence of biglycan and versican in the culture media of fibroblasts cultured from the central airways of 6 asthmatics and 4 healthy subjects [371].

On the other hand, Pini *et al* [132] showed that moderate asthmatics had more immunoreactivity for biglycan and lumican in the smooth muscle layer than severe asthmatics leading them to postulate that this could be protective against excess smooth muscle shortening and hence hyperresponsiveness.

It therefore seems likely that the site of proteoglycan deposition could be at least as important as the degree of expression of these extracellular matrix components.

There is evidence that therapy, particularly corticosteroids, can modulate proteoglycan production. An in vitro study using a human lung fibroblast cell line showed that budesonide, especially if combined with formoterol, counteracted the $TGF\beta_1$-induced upregulation of proteoglycans in the cell medium [460]. It is less clear whether this is what actually happens in the human airway. Immunohistochemistry of the subepithelium in atopic asthmatics exposed to daily low dose allergen challenge over a period of two weeks showed increased biglycan and versican in the group who were receiving concurrent inhaled budesonide [147]. Whether proteoglycan expression is a valid therapeutic target remains to be demonstrated.

7.4 Galectin-3

7.4.1 Summary of findings

Galectin-3 was shown to be expressed in airway epithelial cells and within bronchoalveolar lavage.

Airway epithelial cell expression of galectin-3 showed no difference between asthmatic and non-asthmatic airways. This was assessed by mRNA expression in bronchial brushings, immunoreactivity in endobronchial biopsies and protein levels within the bronchial lavage fluid.

In a group of mild steroid-naive asthmatics, galectin-3 within BALF was shown to increase acutely following bronchial allergen challenge.

7.4.2 Galectin-3 – a relevant contributor to epithelial-mesenchymal cross-talk?

Galectin-3 was investigated to determine whether it could be a relevant factor in the interaction between epithelial cells and underlying mesenchymal cells in fibrotic processes.

In a study involving primary human colon cells, galectin-3 was identified as the main activator of lamina propria fibroblasts when conditioned culture media derived from colonic epithelial cells was compared with unconditioned media [442]. It was upregulated during pulmonary injury in alveolar epithelium from a rat model of irradiation-induced lung injury [461]. Hepatic stellate cells from galectin-3 deficient mice were unable to undergo TGFβ -mediated myofibroblast transformation [439].

Murine asthma models suggest that it may also have a role in eosinophil recruitment and airway hyperresponsiveness induction [445] in addition to airway remodelling (subepithelial fibrosis, smooth muscle thickness and peribronchial angiogenesis) [462].

Within the subjects investigated in this work, no difference could be found in galectin-3's expression in BALF, endobronchial biopsies and bronchial brushings between asthmatics and non-asthmatics. Although levels, as measured by the assays used in these experiments, showed no measurable differences between the groups of subjects, this does not exclude a role for galectin-3 within the micro-environment of the bronchial wall.

Galectin-3 is known to be produced by numerous cell types including epithelia and various inflammatory cells. This work has mainly looked at epithelial production of galectin-3, although the immunochemistry did not reveal any significant staining elsewhere within the biopsy. It could be possible to look at galectin-3 message from whole endobronchial biopsies which would also take into account inflammatory cell contribution.

Considering the results of studies employing galectin-3 knockout mice in a murine asthma model, the lack of a more central role for galectin-3 in stable human asthma is slightly perplexing. A lot of animal research in asthma is hampered by the fact that there is no satisfactory animal model. Murine and other small mammal models reproduce certain aspects of asthma but not the whole disease. The typical murine model involves sensitising the mice, usually to ovalbumin via an intraperitoneal route, and then exposing them to aerosolised ovalbumin after a few days. Despite having numerous advantages over human studies, transposing findings from animal research to humans is fraught with pitfalls. Ovalbumin is not very relevant to human asthma and tolerisation in models attempting to reproduce chronic allergen challenge is a particular

problem. Phenotyping mice through lung function testing and imaging has improved significantly over the years, however there is no real model for severe asthma [463].

7.4.3 Galectin-3 in allergen challenge

Galectin-3 was upregulated in BALF 4 days post-allergen challenge. Further bronchoscopies were not performed to assess later dynamics of BALF galectin-3 levels. However in chronic stable disease, there is no apparent increase when compared to non-asthmatics. It is therefore likely to rise acutely following exposure, with levels then returning to basal levels in the absence of continued stimulation. This does, however, suggest that the mechanisms of chronic disease persistence and acute disease exacerbation may be different.

The rise seen with allergen challenge may be the result of increased epithelial synthesis or enhanced production by other cell types. Levels in lavage may be mainly influenced by epithelial and alveolar macrophage production. The increase seen may be a reflection of increased macrophage numbers [464] which have been described in the immediate aftermath of an allergen challenge. In this particular study, possibly due to the fact that sampling was undertaken 4 days post-challenge, there were no changes in macrophage percentage or total cell counts (data not shown).

Evidence of remodelling in the immediate post-challenge period has been shown in numerous studies [345, 465, 466], so it is possible to imagine that galectin-3 may be playing a role in remodelling processes during this phase. The numerous cytokines and mediators that are affected by allergen exposure are likely to be complex and it is less probable that there is a single agent that is fundamental to the whole process. With these data and the known biological functions of

galectin-3, it could certainly have a relevant role in initiating or promoting subsequent biological processes.

Establishing whether this rise post-challenge has any implications on remodelling, particularly its effect on fibroblasts and/ or lung fibrotic processes would be an interesting next step. The dynamics of BALF galectin-3 levels post-challenge may shed some light as to how important it could be at this point of asthma pathogenesis. This would imply repeated bronchoscopies which may not be acceptable to most subjects. Induced sputum supernatant could be an attractive option if galectin-3 can be detected here.

Galectin-3 has been shown to be important in the transition of fibroblasts to myofibroblasts [439] where there is a change in contractile and synthetic function. A small number of fibroblasts were stimulated with galectin-3 with and without $TGF\beta_2$, but there was no effect of the galectin on its own or any additive effect when in conjunction with TGFβ on proteoglycan output (data not shown). It would still be relevant to look at other extracellular matrix proteins, particularly the ones implicated in post-challenge remodelling – tenascin and collagens. This could provide a mechanism linking galectin-3 with remodelling. The next step would then be to see if medications such as corticosteroids modulate any changes seen.

7.4.4 Other galectins in asthma

Other galectins have been looked at in asthma. Galectin-9 has been shown to be an eosinophil chemoattractant and inducer of T_H1 apoptosis and was upregulated in a murine asthma model [467]. Galectin-10 mRNA was increased in peripheral blood of aspirin-induced asthma [468].

A study in COPD patients [444] looking at galectin-3 and galectin-1, hypothesised that the relative quantities of these two galectins was what differed between COPD patients and matched smokers without COPD. Galectin-1 appears to have a mostly anti-inflammatory role via effects on T cell apoptosis [469]. BALF levels were measured in the same group of patients for whom galectin-3 levels were available (data not shown) but no difference by disease state was found either in absolute values or in proportions to galectin-3.

7.5 Conclusion

Remodelling is resulting from a complex interaction of cells and mediators and it is very likely that differing pathophysiological mechanisms are relevant in different subgroups of asthmatics.

In most lung disease and most certainly in asthma, the epithelium is the first point of contact between the external environment and the constituents of the bronchial wall. Allergens, irritant particles and micro-organisms will initially interact with the mucous lining and then the underlying epithelial cells. Unless appropriately dealt with at this level, chemokines, cytokines and growth factors are released which elicit recruitment of inflammatory cells and responses in the underlying mesenchymal cells. Part of this response involves hypertrophy and hyperplasia of the airway smooth muscle cells as well as increased numbers of fibroblasts and myofibroblasts. The outcome of this is an increased contractile and synthetic capability as well as macroscopically increasing the physical dimensions of the wall. The latter effect arises as a consequence of increased total cell volume and an increase in extracellular matrix deposition.

Figure 7.1 summarises how the work presented here might fit within the bigger picture of airway remodelling. Galectin-3 could be another potential pro-fibrogenic epithelial-derived mediator, the production of which is increased

following allergen challenge. It can promote myofibroblast transformation of fibroblasts which would render them capable of secreting more extracellular matrix protein. Although the central fibroblasts did not show an increased proteoglycan output in vitro when compared to non-asthmatic fibroblasts, they show a response to TGFβ_2, a growth factor known to be increased following epithelial injury. Extracellular matrix not only has a bulking-up effect but also affects airway wall dynamics and cell-cell interactions. It is interesting that central airways from subjects with a fixed airflow obstruction were found to be both thicker and, paradoxically, to have increased luminal areas. This would seem to suggest that airflow obstruction is not an effect of simple static airway narrowing but a more dynamic process relating to wall mechanical properties or arising as an effect of more distal airflow dynamics.

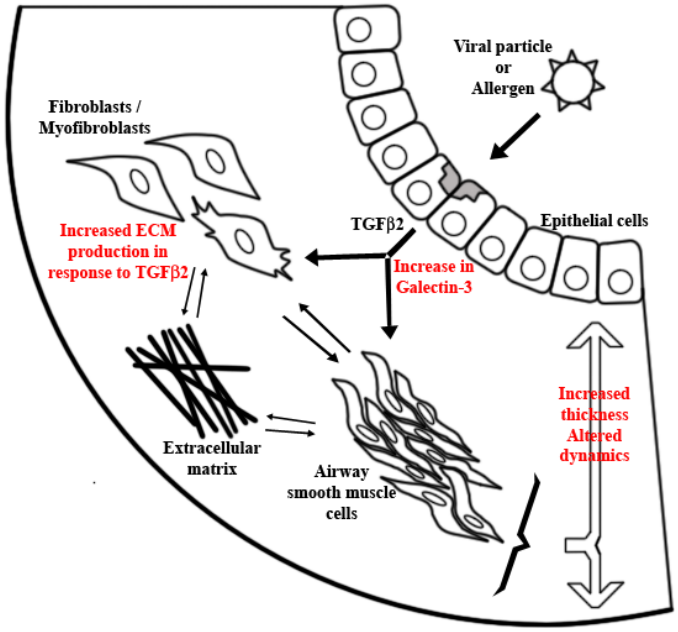

Figure 7.1. Schematic diagram of airway remodelling with points where the topics of this work may be playing a role highlighted in red.

References

1. GINA. *Global Initiative for Asthma: Global Strategy for Asthma Management and Prevention.* 2009; Available from: www.ginasthma.org.

2. Moorman, J.E., et al., *MMWR National Surveillance for Asthma - United States, 1980-2004*. 2007, Centers for Disease Control and Prevention. National Center for Health Statistics. p. 1-14;18-54.

3. *The burden of lung disease: a statistical report from the British Thoracic Society*. 2006, British Thoracic Society.

4. Adcock, I.M., G. Caramori, and K.F. Chung, *New targets for drug development in asthma.* The Lancet, 2008. **372**(9643): p. 1073-1087.

5. Warrier, M.R. and G.K. Khurana Hershey, *Asthma Genetics: Personalizing Medicine.* Journal of Asthma, 2008. **45**(4): p. 257-264.

6. Holloway, J.W., I.A. Yang, and S.T. Holgate, *Genetics of allergic disease.* Journal of Allergy and Clinical Immunology, 2010. **125**(2, Supplement 2): p. S81-S94.

7. Holloway, J.W., I.A. Yang, and S.T. Holgate, *Interpatient variability in rates of asthma progression: Can genetics provide an answer?* Journal of Allergy and Clinical Immunology, 2008. **121**(3): p. 573-579.

8. Arshad, S.H., et al., *Sensitization to Common Allergens and Its Association With Allergic Disorders at Age 4 Years: A Whole Population Birth Cohort Study.* Pediatrics, 2001. **108**(2): p. e33-.

9. Pearce, N., J. Pekkanen, and R. Beasley, *How much asthma is really attributable to atopy?* Thorax, 1999. **54**(3): p. 268-272.

10. Gilliland, F.D., Y.-F. Li, and J.M. Peters, *Effects of Maternal Smoking during Pregnancy and Environmental Tobacco Smoke on Asthma and Wheezing in Children.* Am. J. Respir. Crit. Care Med., 2001. **163**(2): p. 429-436.

11. Flaherman, V. and G.W. Rutherford, *A meta-analysis of the effect of high weight on asthma.* Arch Dis Child, 2006. **91**(4): p. 334-339.

12. Hall, C.B., et al., *Long-term prospective study in children after respiratory syncytial virus infection.* J Pediatr., 1984. **105**(3): p. 358-64.

13. Sigurs, N., et al., *Severe Respiratory Syncytial Virus Bronchiolitis in Infancy and Asthma and Allergy at Age 13.* Am. J. Respir. Crit. Care Med., 2005. **171**(2): p. 137-141.

14. Groskreutz, D.J., et al., *Respiratory Syncytial Virus Induces TLR3 Protein and Protein Kinase R, Leading to Increased Double-Stranded RNA Responsiveness in Airway Epithelial Cells.* J Immunol, 2006. **176**(3): p. 1733-1740.

15. Sly, P.D., M. Kusel, and P.G. Holt, *Do early-life viral infections cause asthma?* Journal of Allergy and Clinical Immunology, 2010. **125**(6): p. 1202-1205.

16. Ackerman, S.J., et al., *Eosinophil Proteins: Structural biology of Charcot-Leyden crystal protein (Galectin-10): new insights into an old protein.* Respiratory Medicine, 2000. **94**(10): p. 1014-1016.

17. Robin, E.D. and N. Lewiston, *Unexpected, unexplained sudden death in young asthmatic subjects.* Chest, 1989. **96**(4): p. 790-793.

18. Sidebotham HJ, R.W., *Asthma deaths; persistent and preventable mortality.* Histopathology, 2003. **43**(2): p. 105 - 17.

19. Oberbeck D, J.W., Petersen C, *[Death in bronchial asthma. Findings and forensic importance].* Versicherungsmedizin, 1993. **45**(2): p. 58 - 63.

20. Dunnill, M., *The pathology of asthma, with special reference to changes in the bronchial mucosa.* Journal of Clinical Pathology, 1960. **13**: p. 27 - 33.

21. Kuwano K, B.C., Pare PD, Bai TR, Wiggs BR, Hogg JC., *Small airways dimensions in asthma and in chronic obstructive pulmonary disease.* American Review of Respiratory Disease, 1993. **148**(5): p. 1220 - 5.

22. Naylor, B., *The shedding of the mucosa of the bronchial tree in asthma.* Thorax, 1962. **17**: p. 69 - 72.

23. Takizawa, T. and W. Thurlbeck, *Muscle and mucous gland size in the major bronchi of patients with chronic bronchitis, asthma and asthmatic bronchitis.* The American Review of Respiratory Disease, 1971. **104**(3): p. 331 - 6.

24. Kay, A.B., *The role of eosinophils in the pathogenesis of asthma.* Trends in Molecular Medicine, 2005. **11**(4): p. 148-152.

25. Azzawi M, J.P., Majumdar S, Kay AB, Jeffery PK, *T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis.* American Review of Respiratory Disease, 1992. **145**(6): p. 1477 - 82.

26. Bradding, P., A.F. Walls, and S.T. Holgate, *The role of the mast cell in the pathophysiology of asthma.* Journal of Allergy and Clinical Immunology, 2006. **117**(6): p. 1277-1284.

27. Carroll, N.G., S. Mutavdzic, and A.L. James, *Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma.* Thorax, 2002. **57**(8): p. 677-682.

28. Wenzel, S.E., et al., *Evidence That Severe Asthma Can Be Divided Pathologically into Two Inflammatory Subtypes with Distinct Physiologic and Clinical Characteristics.* Am. J. Respir. Crit. Care Med., 1999. **160**(3): p. 1001-1008.

29. Louis, R. and R. Djukanovic, *Is the neutrophil a worthy target in severe asthma and chronic obstructive pulmonary disease?* Clinical & Experimental Allergy, 2006. **36**(5): p. 563-567.

30. Wilson, J.W. and X. Li, *The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway.* Clinical And Experimental Allergy: Journal Of The British Society For Allergy And Clinical Immunology, 1997. **27**(4): p. 363-371.

31. Davies, D., *The bronchial epithelium in chronic and severe asthma.* Current Allergy and Asthma Reports, 2001. **1**(2): p. 127 - 33.

32. Laitinen LA, L.A., Altraja A, Virtanen I, Kampe M, Simonsson BG, Karlsson SE, Hakansson L, Venge P, Sillastu H. , *Bronchial biopsy findings in intermittent or "early" asthma.* Journal of Allergy and Clinical Immunology, 1996. **98**(5 Pt 2): p. S3 -6.

33. Ebina M, T.T., Chiba T, Motomiya M., *Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study.* The American Review of Respiratory Disease, 1993. **148**(3): p. 720 -6.

34. Hammad, H. and B.N. Lambrecht, *Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation.* Journal of Allergy and Clinical Immunology, 2006. **118**(2): p. 331-336.

35. Lambrecht, B.N. and H. Hammad, *Biology of Lung Dendritic Cells at the Origin of Asthma.* Immunity, 2009. **31**(3): p. 412-424.

36. Pene, J., et al., *IgE Production by Normal Human Lymphocytes is Induced by Interleukin 4 and Suppressed by Interferons gamma and alpha and Prostaglandin E2.* PNAS, 1988. **85**(18): p. 6880-6884.

37. Punnonen, J., et al., *Interleukin 13 Induces Interleukin 4-Independent IgG4 and IgE Synthesis and CD23 Expression by Human B Cells.* PNAS, 1993. **90**(8): p. 3730-3734.

38. Wardlaw, A., *Molecular basis for selective eosinophil trafficking in asthma: A multistep paradigm.* The Journal of Allergy and Clinical Immunology, 1999. **104**(5): p. 917 - 26.

39. Rivera, J. and A.M. Gilfillan, *Molecular regulation of mast cell activation.* Journal of Allergy and Clinical Immunology, 2006. **117**(6): p. 1214-1225.

40. Bousquet, J., et al., *Eosinophilic inflammation in asthma.* N Engl J Med, 1990. **323**(15): p. 1033-9.

41. Kips, J.C., K.G. Tournoy, and R.A. Pauwels, *New anti-asthma therapies: suppression of the effect of interleukin (IL)-4 and IL-5.* Eur Respir J, 2001. **17**(3): p. 499-506.

42. Busse, W.W. and R.F. Lemanske, *Asthma.* N Engl J Med, 2001. **344**(5): p. 350-362.

43. Ebner, C., et al., *Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients.* J Immunol, 1995. **154**(4): p. 1932-1940.

44. Humbert, M., et al., *IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity.* Am. J. Respir. Crit. Care Med., 1996. **154**(5): p. 1497-1504.

45. Truyen, E., et al., *Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients.* Thorax, 2006. **61**(3): p. 202-208.

46. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties.* Annual Review of Immunology, 1989. **7**(1): p. 145-173.

47. Tang, M.K., AS. Thorburn, J. Hill, DJ., *Reduced interferon-gamma secretion in neonates and subsequent atopy.* Lancet, 1994. **344**(8928): p. 983 - 5.

48. Strachan, D., *Hay fever, hygiene and household size.* British Medical Journal, 1989. **299**: p. 1259 - 60.

49. Bloomfield, S.F., et al., *Too clean, or not too clean: the Hygiene Hypothesis and home hygiene.* Clinical & Experimental Allergy, 2006. **36**(4): p. 402-425.

50. von Mutius, E., *Allergies, infections and the hygiene hypothesis - The epidemiological evidence.* Immunobiology, 2007. **212**(6): p. 433-439.

51. van Oosterhout, A.J.M. and A.C. Motta, *Th1/Th2 paradigm: not seeing the forest for the trees?* Eur Respir J, 2005. **25**(4): p. 591-593.

52. Court, C.S., D.G. Cook, and D.P. Strachan, *Comparative epidemiology of atopic and non-atopic wheeze and diagnosed asthma in a national sample of English adults.* Thorax, 2002. **57**(11): p. 951-7.

53. Mackenney, J., et al., *Prevalence of asthma, atopy and bronchial hyperresponsiveness and their interrelation in a semi-rural area of Chile.* Int J Tuberc Lung Dis, 2005. **9**(11): p. 1288-93.

54. Eduard, W., et al., *Atopic and non-atopic asthma in a farming and a general population.* American Journal of Industrial Medicine, 2004. **46**(4): p. 396-399.

55. Jayaratnam, A., C.J. Corrigan, and T.H. Lee, *The continuing enigma of non-atopic asthma.* Clinical & Experimental Allergy, 2005. **35**(7): p. 835-837.

56. Renauld, J.C., *New insights into the role of cytokines in asthma.* J Clin Pathol, 2001. **54**(8): p. 577-589.

57. Menz, G., et al., *Molecular concepts of IgE-initiated inflammation in atopic and nonatopic asthma.* Allergy, 1998. **53**(45 Suppl): p. 15-21.

58. Broide, D.H., *Immunologic and inflammatory mechanisms that drive asthma progression to remodeling.* Journal of Allergy and Clinical Immunology, 2008. **121**(3): p. 560-570.

59. Ulrik, C.S. and V. Backer, *Nonreversible airflow obstruction in life-long nonsmokers with moderate to severe asthma.* Eur Respir J, 1999. **14**(4): p. 892-896.

60. McFadden, E.R., Jr., *A Century of Asthma.* Am. J. Respir. Crit. Care Med., 2004. **170**(3): p. 215-221.

61. Bento, A.M. and M.B. Hershenson, *Airway remodeling: potential contributions of subepithelial fibrosis and airway smooth muscle hypertrophy/hyperplasia to airway narrowing in asthma.* Allergy Asthma Proc, 1998. **19**(6): p. 353-8.

62. Bucchieri, F., et al., *Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis.* American Journal Of Respiratory Cell And Molecular Biology, 2002. **27**(2): p. 179-185.

63. Holgate, S.T., *Epithelium dysfunction in asthma.* Journal of Allergy and Clinical Immunology, 2007. **120**(6): p. 1233-1244.

64. Hackett, T.-L., et al., *Induction of Epithelial-Mesenchymal Transition in Primary Airway Epithelial Cells from Patients with Asthma by Transforming Growth Factor-β1.* Am. J. Respir. Crit. Care Med., 2009. **180**(2): p. 122-133.

65. Brewster, C.E.P., et al., *Myofibroblasts and subendothelial fibrosis in brochial asthma.* American Journal Of Respiratory Cell And Molecular Biology, 1990. **3**: p. 507 - 511.

66. Keenan, K.P., T.S. Wilson, and E.M. McDowell, *Regeneration of hamster tracheal epithelium after mechanical injury. IV. Histochemical, immunocytochemical and ultrastructural studies.* Virchows Archiv. B, Cell pathology including molecular pathology, 1983. **43**(3): p. 213-40.

67. Shahana, S., et al., *Ultrastructure of bronchial biopsies from patients with allergic and non-allergic asthma.* Respiratory Medicine, 2005. **99**(4): p. 429-443.

68. Dunnill, M.S., *The pathology of asthma with special reference to changes in the bronchial mucosa.* Journal of Clinical Pathology, 1960. **13**(1): p. 27-33.

69. Vignola, A.M., et al., *Increased expression of heat shock protein 70 on airway cells in asthma and chronic bronchitis.* American Journal Of Respiratory Cell And Molecular Biology, 1995. **13**(6): p. 683-91.

70. Puddicombe, S.M., et al., *Involvement of the epidermal growth factor receptor in epithelial repair in asthma.* FASEB J., 2000. **14**(10): p. 1362-1374.

71. Hamilton, L.M., et al., *The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma.* Clinical & Experimental Allergy, 2003. **33**(2): p. 233-240.

72. Vignola, A.M., et al., *Proliferation and activation of bronchial epithelial cells in corticosteroid-dependent asthma.* Journal of Allergy and Clinical Immunology, 2001. **108**(5): p. 738-746.

73. Wark, P.A.B., et al., *Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus.* J. Exp. Med., 2005. **201**(6): p. 937-947.

74. de Boer, W.I., et al., *Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation.* Canadian Journal Of Physiology And Pharmacology, 2008. **86**(3): p. 105-12.

75. Puddicombe, S.M., et al., *Increased Expression of p21waf Cyclin-Dependent Kinase Inhibitor in Asthmatic Bronchial Epithelium.* Am. J. Respir. Cell Mol. Biol., 2003. **28**(1): p. 61-68.

76. Kicic, A., et al., *Intrinsic Biochemical and Functional Differences in Bronchial Epithelial Cells of Children with Asthma.* Am. J. Respir. Crit. Care Med., 2006. **174**(10): p. 1110-1118.

77. Ordonez, C.L., et al., *Mild and Moderate Asthma Is Associated with Airway Goblet Cell Hyperplasia and Abnormalities in Mucin Gene Expression.* Am. J. Respir. Crit. Care Med., 2001. **163**(2): p. 517-523.

78. Carroll, N., et al., *The structure of large and small airways in nonfatal and fatal asthma.* American Review of Respiratory Disease, 1993. **147**(2): p. 405 - 410.

79. Aikawa, T., et al., *Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack.* Chest, 1992. **101**(4): p. 916-921.

80. Kirkham, S., et al., *Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B.* Biochem. J., 2002. **361**(3): p. 537-546.

81. Sheehan, J.K., et al., *Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug.* Biochem. J., 1999. **338**(2): p. 507-513.

82. Rose, M.C. and J.A. Voynow, *Respiratory Tract Mucin Genes and Mucin Glycoproteins in Health and Disease.* Physiol. Rev., 2006. **86**(1): p. 245-278.

83. Thornton, D.J., K. Rousseau, and M.A. McGuckin, *Structure and Function of the Polymeric Mucins in Airways Mucus.* Annual Review of Physiology, 2008. **70**(1): p. 459-486.

84. Rogers, D.F., *Airway mucus hypersecretion in asthma: an undervalued pathology?* Current Opinion in Pharmacology, 2004. **4**(3): p. 241-250.

85. Holgate, S.T., et al., *Epithelial-mesenchymal interactions in the pathogenesis of asthma.* Journal of Allergy and Clinical Immunology, 2000. **105**(2 Pt 1): p. 193 - 204.

86. Davies, D.E., et al., *Airway remodeling in asthma: New insights.* Journal of Allergy and Clinical Immunology, 2003. **111**(2): p. 215-225.

87. Sacco, O., et al., *Epithelial cells and fibroblasts: structural repair and remodelling in the airways.* Paediatric Respiratory Reviews, 2004. **5**(Supplement 1): p. S35-S40.

88. Roche, W., et al., *Subepithelial fibrosis in the bronchi of asthmatics.* The Lancet, 1989. **333**(8637): p. 520-524.

89. Hoshino, M., Y. Nakamura, and J. Sim, *Expression of growth factors and remodelling of the airway wall in bronchial asthma.* Thorax, 1998. **53**(1): p. 21-27.

90. Laitinen, L.A., et al., *Bronchial biopsy findings in intermittent or "early" asthma.* Journal of Allergy and Clinical Immunology, 1996. **98**(5 Pt 2): p. S3 -6.

91. Altraja, A., et al., *Expression of laminins in the airways in various types of asthmatic patients: a morphometric study.* American Journal Of Respiratory Cell And Molecular Biology, 1996. **15**(4): p. 482-8.

92. Liesker, J.J., et al., *Reticular basement membrane in asthma and COPD: similar thickness, yet different composition.* International Journal of Chronic Obstructive Pulmonary Disease, 2009. **4**: p. 127-135.

93. Payne, D.N.R., et al., *Early Thickening of the Reticular Basement Membrane in Children with Difficult Asthma.* Am. J. Respir. Crit. Care Med., 2003. **167**(1): p. 78-82.

94. Barbato, A., et al., *Epithelial Damage and Angiogenesis in the Airways of Children with Asthma.* Am. J. Respir. Crit. Care Med., 2006. **174**(9): p. 975-981.

95. Pohunek, P., et al., *Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma.* Pediatric Allergy and Immunology, 2005. **16**(1): p. 43-51.

96. Bai, T.R., et al., *The Effect of Age and Duration of Disease on Airway Structure in Fatal Asthma.* Am. J. Respir. Crit. Care Med., 2000. **162**(2): p. 663-669.

97. Chetta, A., et al., *Airways Remodeling Is a Distinctive Feature of Asthma and Is Related to Severity of Disease.* Chest, 1997. **111**(4): p. 852-857.

98. Siddiqui, S., et al., *Airway hyperresponsiveness is dissociated from airway wall structural remodeling.* Journal of Allergy and Clinical Immunology, 2008. **122**(2): p. 335-341.e3.

99. Amin, K., et al., *Inflammation and Structural Changes in the Airways of Patients with Atopic and Nonatopic Asthma.* Am. J. Respir. Crit. Care Med., 2000. **162**(6): p. 2295-2301.

100. Braunstahl, G.-J., et al., *Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways.* Clinical & Experimental Allergy, 2003. **33**(5): p. 579-587.

101. Milanese, M., et al., *On the functional consequences of bronchial basement membrane thickening.* J Appl Physiol, 2001. **91**(3): p. 1035-1040.

102. Sohn, S.-W., et al., *Atopy May Be an Important Determinant of Subepithelial Fibrosis in Subjects with Asymptomatic Airway Hyperresponsiveness.* J Korean Med Sci, 2008. **23**(3): p. 390-396.

103. Hirst, S.J., et al., *Proliferative aspects of airway smooth muscle.* Journal of Allergy and Clinical Immunology, 2004. **114**(2, Supplement 1): p. S2-S17.

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

105. James, A.L., P.D. Pare, and J.C. Hogg, *The mechanics of airway narrowing in asthma.* American Review of Respiratory Disease, 1989. **139**(1): p. 242 - 246.

106. Saetta, M., et al., *Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma.* American Review of Respiratory Disease, 1991. **143**(1): p. 138 - 43.

107. Benayoun, L., et al., *Airway Structural Alterations Selectively Associated with Severe Asthma.* Am. J. Respir. Crit. Care Med., 2003. **167**(10): p. 1360-1368.

108. Woodruff, P.G., et al., *Hyperplasia of Smooth Muscle in Mild to Moderate Asthma without Changes in Cell Size or Gene Expression.* Am. J. Respir. Crit. Care Med., 2004. **169**(9): p. 1001-1006.

109. Ebina, M., et al., *Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study.* The American Review of Respiratory Disease, 1993. **148**(3): p. 720 -6.

110. Johnson, P.R.A., et al., *Airway Smooth Muscle Cell Proliferation Is Increased in Asthma.* Am. J. Respir. Crit. Care Med., 2001. **164**(3): p. 474-477.

111. Freyer, A.M., S.R. Johnson, and I.P. Hall, *Effects of Growth Factors and Extracellular Matrix on Survival of Human Airway Smooth Muscle Cells.* Am. J. Respir. Cell Mol. Biol., 2001. **25**(5): p. 569-576.

112. Roth, M., et al., *Dysfunctional Interaction of C/EBPα and the Glucocorticoid Receptor in Asthmatic Bronchial Smooth-Muscle Cells.* N Engl J Med, 2004. **351**(6): p. 560-574.

113. Goldie, R.G., et al., *In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, beta-adrenoceptor agonists and theophylline.* British Journal of Clinical Pharmacology, 1986. **22**(6): p. 669-676.

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

115. Ma, X., et al., *Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects.* Am J Physiol Lung Cell Mol Physiol, 2002. **283**(6): p. L1181-1189.

116. Shore, S.A. and P.E. Moore, *Regulation of β-adrenergic responses in airway smooth muscle.* Respiratory Physiology & Neurobiology, 2003. **137**(2-3): p. 179-195.

117. Brown, R.H., et al., *High-Resolution Computed Tomographic Evaluation of Airway Distensibility and the Effects of Lung Inflation on Airway Caliber in Healthy Subjects and Individuals with Asthma.* Am. J. Respir. Crit. Care Med., 2001. **163**(4): p. 994-1001.

118. Ghaffar, O., et al., *Constitutive and Cytokine-Stimulated Expression of Eotaxin by Human Airway Smooth Muscle Cells.* Am. J. Respir. Crit. Care Med., 1999. **159**(6): p. 1933-1942.

119. John, M., et al., *Human airway smooth muscle cells express and release RANTES in response to T helper 1 cytokines: regulation by T helper 2 cytokines and corticosteroids.* Journal of Immunology, 1997. **158**(4): p. 1841-7.

120. Hakonarson, H., et al., *Autocrine interaction between IL-5 and IL-1β mediates altered responsiveness of atopic asthmatic sensitized airway smooth muscle.* The Journal of Clinical Investigation, 1999. **104**(5): p. 657-667.

121. Elias, J.A., et al., *Cytokine- and virus-stimulated airway smooth muscle cells produce IL-11 and other IL-6-type cytokines.* American Journal of Physiology, 1997. **273**(3 pt 1): p. L648-55.

122. Saunders, M.A., et al., *Release of granulocyte-macrophage colony stimulating factor by human cultured airway smooth muscle cells: suppression by dexamethasone.* British Journal of Pharmacology, 1997. **120**(4): p. 545-546.

123. McKay, S., et al., *Angiotensin II Induces Hypertrophy of Human Airway Smooth Muscle Cells: Expression of Transcription Factors and Transforming Growth Factor-beta 1.* Am. J. Respir. Cell Mol. Biol., 1998. **18**(6): p. 823-833.

124. Wen, F.-Q., et al., *TH2 Cytokine-enhanced and TGF-β-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN-γ and corticosteroids.* Journal of Allergy and Clinical Immunology, 2003. **111**(6): p. 1307-1318.

125. Panettieri, R., Jr, et al., *Activation of cAMP-dependent pathways in human airway smooth muscle cells inhibits TNF-alpha-induced ICAM-1 and VCAM-1 expression and T lymphocyte adhesion.* J Immunol, 1995. **154**(5): p. 2358-2365.

126. Johnson, S.R. and A.J. Knox, *Synthetic functions of airway smooth muscle in asthma.* Trends in Pharmacological Sciences, 1997. **18**(8): p. 288-292.

127. Beasley, R., et al., *Cellular events in the bronchi in mild asthma and after bronchial provocation.* American Review of Respiratory Disease, 1989. **139**(3): p. 806-17.

128. Jeffery, P.K., et al., *Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study.* American Review of Respiratory Disease, 1992. **145**(4 Pt 1): p. 890 - 899.

129. Chu, Hong W., et al., *Collagen Deposition in Large Airways May Not Differentiate Severe Asthma from Milder Forms of the Disease.* Am. J. Respir. Crit. Care Med., 1998. **158**(6): p. 1936-1944.

130. Huang, J.U.N., et al., *Enhanced Proteoglycan Deposition in the Airway Wall of Atopic Asthmatics.* Am. J. Respir. Crit. Care Med., 1999. **160**(2): p. 725-729.

131. de Medeiros Matsushita, M., et al., *Airway proteoglycans are differentially altered in fatal asthma.* The Journal of Pathology, 2005. **207**(1): p. 102-110.

132. Pini, L., et al., *Differences in proteoglycan deposition in the airways of moderate and severe asthmatics.* Eur Respir J, 2007. **29**(1): p. 71-77.

133. Kuwano, K., et al., *Small airways dimensions in asthma and in chronic obstructive pulmonary disease.* American Review of Respiratory Disease, 1993. **148**(5): p. 1220 - 5.

134. Bush, A., *How early do inflammation and remodeling occur?* Allergol Int, 2008. **57**(1): p. 11-9.

135. Fedorov, I.A., et al., *Epithelial stress and structural remodelling in childhood asthma.* Thorax, 2005. **60**(5): p. 389-394.

136. Wagner, E.M., et al., *Peripheral lung resistance in normal and asthmatic subjects.* American Review of Respiratory Disease, 1990. **141**(3): p. 584 - 588.

137. Yanai, M., et al., *Site of airway obstruction in pulmonary disease: direct measurement of intrabronchial pressure.* J Appl Physiol, 1992. **72**(3): p. 1016-1023.

138. Ohrui, T., et al., *Partitioning of pulmonary responses to inhaled methacholine in subjects with asymptomatic asthma.* American Review of Respiratory Disease, 1992. **146**(6): p. 1501-5.

139. Carroll, N., C. Cooke, and A. James, *The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics.* Eur Respir J, 1997. **10**(2): p. 292-300.

140. Shiba, K., et al., *Structural Changes of the Airway Wall Impair Respiratory Function, Even in Mild Asthma*.* Chest, 2002. **122**(5): p. 1622-1626.

141. Minshall, E., et al., *Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma.* Am. J. Respir. Cell Mol. Biol., 1997. **17**(3): p. 326-333.

142. Boulet, L.-P., et al., *Bronchial Subepithelial Fibrosis Correlates With Airway Responsiveness to Methacholine.* Chest, 1997. **112**(1): p. 45-52.

143. Niimi, A., et al., *Relationship of Airway Wall Thickness to Airway Sensitivity and Airway Reactivity in Asthma.* Am. J. Respir. Crit. Care Med., 2003. **168**(8): p. 983-988.

144. McParland, B.E., P.T. Macklem, and P.D. Pare, *Airway wall remodeling: friend or foe?* J Appl Physiol, 2003. **95**(1): p. 426-434.

145. Lundgren, R., et al., *Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids.* Eur Respir J, 1988. **1**(10): p. 883-9.

146. Laitinen, L.A., A. Laitinen, and T. Haahtela, *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, 1992. **90**(1): p. 32-42.

147. de Kluijver, J., et al., *Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma.* Clinical & Experimental Allergy, 2005. **35**(10): p. 1361-1369.

148. Trigg, C.J., et al., *Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma.* Am J Respir Crit Care Med, 1994. **150**(1): p. 17-22.

149. Olivieri, D., et al., *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, 1997. **155**(6): p. 1864-71.

150. Ward, C., et al., *Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma.* Thorax, 2002. **57**(4): p. 309-316.

151. Capraz, F., et al., *The effect of inhaled budesonide and formoterol on bronchial remodeling and HRCT features in young asthmatics.* Lung, 2007. **185**(2): p. 89-96.

152. Hoshino, et al., *Inhaled corticosteroid reduced lamina reticularis of the basement membrane by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma.* Clinical & Experimental Allergy, 1998. **28**(5): p. 568-577.

153. Boulet, L.P., et al., *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., 2000. **162**(4): p. 1308-1313.

154. Lee, S.Y., et al., *Inhaled corticosteroid prevents the thickening of airway smooth muscle in murine model of chronic asthma.* Pulmonary Pharmacology & Therapeutics, 2008. **21**(1): p. 14-19.

155. Miller, M., et al., *Corticosteroids prevent myofibroblast accumulation and airway remodeling in mice.* Am J Physiol Lung Cell Mol Physiol, 2006. **290**(1): p. L162-169.

156. Cho, J.Y., et al., *Combination of corticosteroid therapy and allergen avoidance reverses allergen-induced airway remodeling in mice.* Journal of Allergy and Clinical Immunology, 2005. **116**(5): p. 1116-1122.

157. Hoshino, M., et al., *Inhaled corticosteroids decrease vascularity of the bronchial mucosa in patients with asthma.* Clinical & Experimental Allergy, 2001. **31**(5): p. 722-730.

158. Mauad, T., E.H. Bel, and P.J. Sterk, *Asthma therapy and airway remodeling.* Journal of Allergy and Clinical Immunology, 2007. **120**(5): p. 997-1009.

159. Bel, E.H., *Bronchial Thermoplasty: Has the Promise Been Met?* Am. J. Respir. Crit. Care Med. **181**(2): p. 101-102.

160. Gabbiani, G., G.B. Ryan, and G. Majne, *Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction.* Experientia, 1971. **27**(5): p. 549 - 550.

161. Tomasek, J.J., et al., *Myofibroblasts and mechano-regulation of connective tissue remodelling.* Nat Rev Mol Cell Biol, 2002. **3**(5): p. 349-363.

162. Darby, I., O. Skalli, and G. Gabbiani, *Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing.* Laboratory Investigation, 1990. **63**(1): p. 21-29.

163. Hinz, B., et al., *Myofibroblast Development Is Characterized by Specific Cell-Cell Adherens Junctions.* Mol. Biol. Cell, 2004. **15**(9): p. 4310-4320.

164. Hinz, B., *Formation and Function of the Myofibroblast during Tissue Repair.* J Invest Dermatol, 2007. **127**(3): p. 526-537.

165. Abe, R., et al., *Peripheral Blood Fibrocytes: Differentiation Pathway and Migration to Wound Sites.* J Immunol, 2001. **166**(12): p. 7556-7562.

166. Bellini, A. and S. Mattoli, *The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses.* Lab Invest, 2007. **87**(9): p. 858-870.

167. Wu, Z., et al., *Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse.* Respiratory Research, 2007. **8**(1): p. 1.

168. Willis, B.C., R.M. duBois, and Z. Borok, *Epithelial Origin of Myofibroblasts during Fibrosis in the Lung.* Proc Am Thorac Soc, 2006. **3**(4): p. 377-382.

169. Rajkumar, V., et al., *Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a*

myofibroblast lineage in fibrosis. Arthritis Research & Therapy, 2005. **7**(5): p. R1113 - R1123.

170. Olivier De Wever, M.M., *Role of tissue stroma in cancer cell invasion.* The Journal of Pathology, 2003. **200**(4): p. 429-447.

171. Hu, B., Z. Wu, and S.H. Phan, *Smad3 Mediates Transforming Growth Factor-β-Induced α-Smooth Muscle Actin Expression.* Am. J. Respir. Cell Mol. Biol., 2003. **29**(3): p. 397-404.

172. Wilkes, M.C., et al., *Transforming Growth Factor-β Activation of Phosphatidylinositol 3-Kinase Is Independent of Smad2 and Smad3 and Regulates Fibroblast Responses via p21-Activated Kinase-2.* Cancer Res, 2005. **65**(22): p. 10431-10440.

173. Hinz, B., *Masters and servants of the force: The role of matrix adhesions in myofibroblast force perception and transmission.* European Journal of Cell Biology, 2006. **85**(3-4): p. 175-181.

174. Giannone, G. and M.P. Sheetz, *Substrate rigidity and force define form through tyrosine phosphatase and kinase pathways.* Trends in Cell Biology, 2006. **16**(4): p. 213-223.

175. Wang, J., R. Zohar, and C.A. McCulloch, *Multiple roles of α-smooth muscle actin in mechanotransduction.* Experimental Cell Research, 2006. **312**(3): p. 205-214.

176. Desmouliere, A., et al., *Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar.* American Journal of Pathology, 1995. **146**(1): p. 56 - 66.

177. Zhang, H.-Y. and S.H. Phan, *Inhibition of Myofibroblast Apoptosis by Transforming Growth Factor beta 1.* Am. J. Respir. Cell Mol. Biol., 1999. **21**(6): p. 658-665.

178. Horowitz, J.C., et al., *Activation of the Pro-survival Phosphatidylinositol 3-Kinase/AKT Pathway by Transforming Growth Factor-β1 in Mesenchymal Cells Is Mediated by p38 MAPK-dependent Induction of an Autocrine Growth Factor.* J. Biol. Chem., 2004. **279**(2): p. 1359-1367.

179. Doherty, T. and D. Broide, *Cytokines and growth factors in airway remodeling in asthma.* Current Opinion in Immunology, 2007. **19**(6): p. 676-680.

180. Hinz, B., et al., *The Myofibroblast: One Function, Multiple Origins.* Am J Pathol, 2007. **170**(6): p. 1807-1816.

181. Blobe, G.C., W.P. Schiemann, and H.F. Lodish, *Role of Transforming Growth Factor β in Human Disease.* N Engl J Med, 2000. **342**(18): p. 1350-1358.

182. Sinha, S., et al., *Cellular and extracellular biology of the latent transforming growth factor-β binding proteins.* Matrix Biology, 1998. **17**(8-9): p. 529-545.

183. Garcia-Alvarez, J., et al., *Tissue inhibitor of metalloproteinase-3 is up-regulated by Transforming Growth Factor-β1 in vitro and expressed in fibroblastic foci in vivo in idiopathic pulmonary fibrosis.* Experimental Lung Research, 2006. **32**(5): p. 201 - 214.

184. Xie, S., et al., *Mechanisms of induction of airway smooth muscle hyperplasia by transforming growth factor-beta.* Am J Physiol Lung Cell Mol Physiol, 2007. **293**(1): p. L245-253.

185. Brightling, C., M. Berry, and Y. Amrani, *Targeting TNF-α: A novel therapeutic approach for asthma.* Journal of Allergy and Clinical Immunology, 2008. **121**(1): p. 5-10.

186. Scheurich, P., et al., *Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-alpha: induction of TNF receptors on human T cells and TNF-alpha- mediated enhancement of T cell responses.* J Immunol, 1987. **138**(6): p. 1786-1790.

187. Lukacs, N., et al., *TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation.* J Immunol, 1995. **154**(10): p. 5411-5417.

188. Lassalle, P., et al., *Potential implication of endothelial cells in bronchial asthma.* International Archives of Allergy and Immunology, 1991. **94**(1-4): p. 233-8.

189. Sukkar, M.B., et al., *Tumour necrosis factor-alpha potentiates contraction of human bronchus in vitro.* Respirology, 2001. **6**(3): p. 199-203.

190. Howarth, P.H., et al., *Tumour necrosis factor (TNFα) as a novel therapeutic target in symptomatic corticosteroid dependent asthma.* Thorax, 2005. **60**(12): p. 1012-1018.

191. Sullivan, D.E., et al., *Tumor Necrosis Factor-α Induces Transforming Growth Factor-β1 Expression in Lung Fibroblasts Through the Extracellular Signal-Regulated Kinase Pathway.* Am. J. Respir. Cell Mol. Biol., 2005. **32**(4): p. 342-349.

192. Amrani, Y., et al., *Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells.* American Journal Of Respiratory Cell And Molecular Biology, 1996. **15**(1): p. 55-63.

193. Alberts, B., et al., *Molecular Biology of the Cell*. 4th ed, ed. B. Alberts. 2002: Garland Science.

194. Westergren-Thorsson, G., et al., *Lung fibroblast clones from normal and fibrotic subjects differ in hyaluronan and decorin production and rate of proliferation.* The International Journal of Biochemistry & Cell Biology, 2004. **36**(8): p. 1573-1584.

195. Chakir, J., et al., *Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-β, IL-11, IL-17, and type I and type III collagen expression.* Journal of Allergy and Clinical Immunology, 2003. **111**(6): p. 1293-1298.

196. Iozzo, R.V., *Matrix Proteoglycans: From Molecular Design to Cellular Function.* Annual Review of Biochemistry, 1998. **67**(1): p. 609-652.

197. Huber, H.L. and K.K. Kossler, *The pathology of bronchial asthma.* Archives of Internal Medicine, 1922. **30**: p. 189 - 760.

198. Moreno, R.H., J.C. Hogg, and P.D. Pare, *Mechanics of airway narrowing.* The American Review of Respiratory Disease, 1986. **133**: p. 1171 - 1180.

199. Hirota, S., et al., *Airway smooth muscle excitation-contraction coupling and airway hyperresponsiveness.* Canadian Journal Of Physiology And Pharmacology, 2005. **83**(8-9): p. 725-732.

200. Lambert, R.K., et al., *Functional significance of increased airway smooth muscle in asthma and COPD.* J Appl Physiol, 1993. **74**(6): p. 2771-2781.

201. Boulet, L., M. Belanger, and G. Carrier, *Airway responsiveness and bronchial-wall thickness in asthma with or without fixed airflow obstruction.* American Journal of Respiratory and Critical Care Medicine, 1995. **152**(3): p. 865 - 71.

202. Okazawa, M., et al., *Human airway narrowing measured using high resolution computed tomography.* American Journal of Respiratory and Critical Care Medicine, 1996. **154**(5): p. 1557 - 62.

203. Park, J.W., et al., *High-resolution computed tomography in patients with bronchial asthma: correlation with clinical features, pulmonary functions and bronchial hyperresponsiveness.* Journal of Investigational Allergology and Clinical Immunology, 1997. **7**(3): p. 186 - 92.

204. Awadh, N., et al., *Airway wall thickness in patients with near fatal asthma and control groups: assessment with high resolution computed tomographic scanning.* Thorax, 1998. **53**(4): p. 248-253.

205. Niimi, A., et al., *Airway Wall Thickness in Asthma Assessed by Computed Tomography . Relation to Clinical Indices.* Am. J. Respir. Crit. Care Med., 2000. **162**(4): p. 1518-1523.

206. Little, S.A., et al., *High resolution computed tomographic assessment of airway wall thickness in chronic asthma: reproducibility and relationship with lung function and severity.* Thorax, 2002. **57**(3): p. 247-253.

207. Kasahara, K., et al., *Correlation between the bronchial subepithelial layer and whole airway wall thickness in patients with asthma.* Thorax, 2002. **57**(3): p. 242-246.

208. Shaw, T.J., et al., *Endobronchial ultrasound to assess airway wall thickening: validation in vitro and in vivo.* Eur Respir J, 2004. **23**(6): p. 813-817.

209. Gono, H., et al., *Evaluation of airway wall thickness and air trapping by HRCT in asymptomatic asthma.* Eur Respir J, 2003. **22**(6): p. 965-971.

210. Matsumoto, H., et al., *Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma.* Thorax, 2005. **60**(4): p. 277-281.

211. Backman, K.S., P.A. Greenberger, and R. Patterson, *Airways Obstruction in Patients With Long-term Asthma Consistent With `Irreversible Asthma'.* Chest, 1997. **112**(5): p. 1234-1240.

212. Jang, A.S., et al., *Risk factors related to fixed airway obstruction in patients with asthma after antiasthma treatment.* Ann Allergy Asthma Immunol, 2007. **99**(5): p. 408-12.

213. Contoli, M., et al., *Fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease: 5-year follow-up.* The Journal of Allergy and Clinical Immunology. **125**(4): p. 830-837.

214. Bumbacea, D., et al., *Parameters associated with persistent airflow obstruction in chronic severe asthma.* Eur Respir J, 2004. **24**(1): p. 122-128.

215. Baker, K.M., D.A. Brand, and J. Hen, Jr., *Classifying Asthma: Disagreement Among Specialists.* Chest, 2003. **124**(6): p. 2156-2163.

216. Humbert, M., et al., *Asthma control or severity: that is the question.* Allergy, 2007. **62**(2): p. 95-101.

217. Wenzel, S.E., *Asthma: defining of the persistent adult phenotypes.* The Lancet, 2006. **368**(9537): p. 804-813.

218. Yawn, B.P., et al., *Assessment of Asthma Severity and Asthma Control in Children.* Pediatrics, 2006. **118**(1): p. 322-329.

219. Cowen, M.K., D.B. Wakefield, and M.M. Cloutier, *Classifying Asthma Severity: Objective Versus Subjective Measures.* Journal of Asthma, 2007. **44**(9): p. 711 - 715.

220. Juniper, E.F., et al., *Development and validation of a questionnaire to measure asthma control.* Eur Respir J, 1999. **14**(4): p. 902-907.

221. Bateman, E.D., *Measuring asthma control.* Current Opinion Allergy and CLinical Immunology, 2001. **1**(3): p. 211 - 216.

222. Moy, M.L., et al., *Clinical Predictors of Health-related Quality of Life Depend on Asthma Severity.* Am. J. Respir. Crit. Care Med., 2001. **163**(4): p. 924-929.

223. Wever, A.M., et al., *Clinical evaluation of five spirometers. Monaghan M403, Pneumoscreen, Spirotron, Vicatest and Vitalograph.* European Journal of Respiratory Disease, 1981. **62**(2): p. 127 - 137.

224. Mandal, N.G., *Respirometers including spirometer, pneumotachograph and peak flow meter.* Anaesthesia & intensive care medicine, 2006. **7**(1): p. 1-5.

225. Enright, P.L., K.C. Beck, and D.L. Sherrill, *Repeatability of Spirometry in 18,000 Adult Patients.* Am. J. Respir. Crit. Care Med., 2004. **169**(2): p. 235-238.

226. Burrows, B., *Course and prognosis of patients with chronic airways obstruction.* Chest, 1980. **77**(2): p. 250-251.

227. Panizza, J.A., et al., *Mortality and airflow obstruction in asthma: a 17-year follow-up study.* Internal Medicine Journal, 2006. **36**(12): p. 773 - 780.

228. Aburuz, S., et al., *Relationship Between Lung Function and Asthma Symptoms in Patients with Difficult to Control Asthma.* Journal of Asthma, 2005. **42**(10): p. 859 - 864.

229. Teeter, J.G. and E.R. Bleecker, *Relationship Between Airway Obstruction and Respiratory Symptoms in Adult Asthmatics.* Chest, 1998. **113**(2): p. 272-277.

230. Busse, W.B. and S.T. Holgate, *Asthma and rhinitis*. Second ed. Vol. 2. 2000: Blackwell Science Inc. 1242.

231. Miller, M.R., et al., *Standardisation of spirometry.* Eur Respir J, 2005. **26**(2): p. 319-338.

232. Brusasco, V., R. Crapo, and G. Viegi, *Coming together: the ATS/ERS consensus on clinical pulmonary function testing.* Eur Respir J, 2005. **26**(1): p. 1-2.

233. ECSC, *Standardized lung function testing. Report working party.* Bulletin Europeen de Physiopathologie Respiratoire, 1983. **19**(Suppl 5): p. 1 - 95.

234. Roca, J., et al., *References values for forced spirometry. Group of the European Community Respiratory Health Survey.* Eur Respir J, 1998. **11**(6): p. 1354-1362.

235. Crapo, R.O., *The role of reference values in interpreting lung function tests.* Eur Respir J, 2004. **24**(3): p. 341-342.

236. Lorber, D.B., W. Kaltenborn, and B. Burrows, *Responses to isoproterenol in a general population.* The American Review of Respiratory Disease, 1978. **118**(5): p. 855 - 861.

237. Dales, R.E., et al., *Clinical interpretation of airway response to a bronchodilator. Epidemiologic considerations.* The American Review of Respiratory Disease, 1988. **138**(2): p. 317 - 320.

238. Tweeddale, P.M., F. Alexander, and G.J. McHardy, *Short term variability in FEV1 and bronchodilator responsiveness in patients with obstructive ventilatory defects.* Thorax, 1987. **42**(7): p. 487-490.

239. Johannessen, A., et al., *Post-Bronchodilator Spirometry Reference Values in Adults and Implications for Disease Management.* Am. J. Respir. Crit. Care Med., 2006. **173**(12): p. 1316-1325.

240. Appleton, S.L., et al., *Spirometric criteria for asthma: Adding further evidence to the debate.* Journal of Allergy and Clinical Immunology, 2005. **116**(5): p. 976-982.

241. Barnes, P.J., et al., *Partial flow-volume curves to measure bronchodilator dos-response curves in normal humans.* J Appl Physiol, 1981. **50**(6): p. 1193-1197.

242. Borg, B.M., et al., *Bronchodilator reversibility testing: laboratory practices in Australia and New Zealand.* Medical Journal of Australia, 2004. **180**(12): p. 605 - 606.

243. Newman, S.P., et al., *Pressurised aerosol deposition in the human lung with and without an "open" spacer device.* Thorax, 1989. **44**(9): p. 706-10.

244. Brand, P.L., et al., *Interpretation of bronchodilator response in patients with obstructive airways disease. The Dutch Chronic Non-Specific Lung Disease (CNSLD) Study Group.* Thorax, 1992. **47**(6): p. 429-436.

245. Yancey, S.W. and H.G. Ortega, *Retrospective characterization of airway reversibility in patients with asthma responsive to bronchodilators.* Current Medical Research and Opinion, 2007. **23**(12): p. 3205 - 3207.

246. Lehmann, S., et al., *Bronchodilator reversibility testing in an adult general population; the importance of smoking and anthropometrical variables on the response to a β2-agonist.* Pulmonary Pharmacology & Therapeutics, 2006. **19**(4): p. 272-280.

247. Hunter, C.J., et al., *A Comparison of the Validity of Different Diagnostic Tests in Adults With Asthma*.* Chest, 2002. **121**(4): p. 1051-1057.

248. E.A.A.C.I., *1. Pathophysiology of skin tests.* Allergy, 1989. **44**(s10): p. 13-21.

249. Burney, P., et al., *The distribution of total and specific serum IgE in the European Community Respiratory Health Survey.* Journal of Allergy and Clinical Immunology, 1997. **99**: p. 314 - 322.

250. Raukas-Kivioja, A., et al., *Allergic sensitization among adults in Tallinn, Estonia.* Clinical & Experimental Allergy, 2003. **33**(10): p. 1342-1348.

251. Pallasaho, P., et al., *Degree and clinical relevance of sensitization to common allergens among adults: a population study in Helsinki, Finland.* Clinical & Experimental Allergy, 2006. **36**(4): p. 503-509.

252. Sunyer, J., et al., *Risk factors for asthma in young adults. Spanish Group of the European Community Respiratory Health Survey.* Eur Respir J, 1997. **10**(11): p. 2490-2494.

253. Burrows, B., et al., *Association of asthma with serum IgE levels and skin-test reactivity to allergens.* New England Journal of Medicine, 1989. **320**(5): p. 271 - 277.

254. Peat, J.K. and A.J. Woolcock, *Sensitivity to common allergens: relation to respiratory symptoms and bronchial hyper-responsiveness in children from three different climatic areas of Australia.* Clinical & Experimental Allergy, 1991. **21**(5): p. 573 - 581.

255. Sporik, R., et al., *Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study.* The New England Journal of Medicine, 1990. **323**(8): p. 502 - 507.

256. Sears, M.R., et al., *Atopy in childhood. I. Gender and allergen related risks for development of hay fever and asthma.* Clinical & Experimental Allergy, 1993. **23**(11): p. 941 - 948.

257. Sears, M.R., et al., *A Longitudinal, Population-Based, Cohort Study of Childhood Asthma Followed to Adulthood.* N Engl J Med, 2003. **349**(15): p. 1414-1422.

258. E.A.A.C.I., *2. Methods for skin testing.* Allergy, 1989. **44**(s10): p. 22-30.

259. Jorens, P.G., P.A. Vermeire, and A.G. Herman, *L-arginine-dependent nitric oxide synthase: a new metabolic pathway in the lung and airways.* Eur Respir J, 1993. **6**(2): p. 258 - 266.

260. Hamid, Q., et al., *Induction of nitric oxide synthase in asthma.* Lancet, 1993. **342**(8886-8887): p. 1510 - 1513.

261. Ricciardolo, F.L.M., et al., *Nitric Oxide in Health and Disease of the Respiratory System.* Physiol. Rev., 2004. **84**(3): p. 731-765.

262. Gustafsson, L.E., et al., *Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans.* Biochemical and Biophysical Research Communications, 1991. **181**(2): p. 852-857.

263. Alving, K., E. Weitzberg, and J.M. Lundberg, *Increased amount of nitric oxide in exhaled air of asthmatics.* Eur Respir J, 1993. **6**(9): p. 1368-1370.

264. Kharitonov, S.A., et al., *Exhaled Nitric Oxide Is Increased in Asthma.* Chest, 1995. **107**(3_Supplement): p. 156S-157.

265. Massaro, A.F., et al., *Expired nitric oxide levels during treatment of acute asthma.* Am. J. Respir. Crit. Care Med., 1995. **152**(2): p. 800 - 803.

266. Kharitonov, S.A., et al., *Increased nitric oxide in exhaled air of asthmatic patients.* Lancet, 1994. **343**(8890): p. 133 - 135.

267. Persson, M.G., et al., *Single-breath nitric oxide measurements in asthmatic pateints and smokers.* Lancet, 1994. **343**(8890): p. 146 - 147.

268. Jatakanon, A., et al., *Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma.* Thorax, 1998. **53**(2): p. 91 - 95.

269. Dupont, L.J., et al., *Exhaled nitric oxide correlates with airway hyperresponsiveness in steroid-naive patients with mild asthma.* Am. J. Respir. Crit. Care Med., 1998. **157**(3 Pt 1): p. 894 - 898.

270. Franklin, P.J., et al., *Exhaled nitric oxide and asthma: complex interactions between atopy, airway responsiveness, and symptoms in a community population of children.* Thorax, 2003. **58**(12): p. 1048-1052.

271. Franklin, P.J., et al., *Measuring Exhaled Nitric Oxide Levels in Adults: The Importance of Atopy and Airway Responsiveness.* Chest, 2004. **126**(5): p. 1540-1545.

272. Yates, D., et al., *Effect of a nitric oxide inhibitor and a glucocorticoid on exhaled nitric oxide.* Am. J. Respir. Crit. Care Med., 1995. **152**(3): p. 892 - 896.

273. Silkoff, P.E., et al., *Exhaled nitric oxide and bronchial reactivity during and after inhaled beclomethasone in mild asthma.* Journal of Asthma, 1998. **35**(6): p. 473 – 479.

274. Smith, A.D., et al., *Diagnosing Asthma: Comparisons between Exhaled Nitric Oxide Measurements and Conventional Tests.* Am. J. Respir. Crit. Care Med., 2004. **169**(4): p. 473–478.

275. Smith, A.D., et al., *Use of Exhaled Nitric Oxide Measurements to Guide Treatment in Chronic Asthma.* N Engl J Med, 2005. **352**(21): p. 2163–2173.

276. Beck-Ripp, J., et al., *Changes of exhaled nitric oxide during steroid treatment of childhood asthma.* Eur Respir J, 2002. **19**(6): p. 1015–1019.

277. Jones, S.L., et al., *The Predictive Value of Exhaled Nitric Oxide Measurements in Assessing Changes in Asthma Control.* Am. J. Respir. Crit. Care Med., 2001. **164**(5): p. 738–743.

278. Archer, S., *Measurement of nitric oxide in biological models.* FASEB J., 1993. **7**(2): p. 349–360.

279. Fontijin, A., A.J. Sabadell, and R.J. Ronco, *Homogenous chemiluminescent measurement of nitric oxide with ozone: implications for continuous selective monitoring of gaseous pollutants.* Analytical chemistry, 1970. **42**(6): p. 575 – 579.

280. Borrill, Z., et al., *A comparison of exhaled nitric oxide measurements performed using three different analysers.* Respiratory Medicine, 2006. **100**(8): p. 1392–1396.

281. Muller, K.C., et al., *Comparison of exhaled nitric oxide analysers.* Respiratory Medicine, 2005. **99**(5): p. 631–637.

282. Silkoff, P.E., et al., *Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide.* Am. J. Respir. Crit. Care Med., 1997. **155**(1): p. 260 – 267.

283. Kimberly, B., et al., *Nasal contribution to exhaled nitric oxide at rest and during breathholding in humans.* Am. J. Respir. Crit. Care Med., 1996. **153**(2): p. 829 – 836.

284. Kharitonov, S.A. and P.J. Barnes, *Nasal contribution to exhaled nitric oxide during exhalation against resistance or during breath holding.* Thorax, 1997. **52**(6): p. 540 – 544.

285. Dupont, L.J., M.G. Demedts, and G.M. Verleden, *Prospective evaluation of the validity of exhaled nitric oxide for the diagnosis of asthma.* Chest, 2003. **123**(3): p. 751-756.

286. ATS/ERS, *ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005.* Am. J. Respir. Crit. Care Med., 2005. **171**(8): p. 912-930.

287. Cockcroft, D.W. and B.E. Davis, *Mechanisms of airway hyperresponsiveness.* Journal of Allergy and Clinical Immunology, 2006. **118**(3): p. 551-559.

288. Birnbaum, S. and T.J. Barreiro, *Methacholine Challenge Testing: Identifying Its Diagnostic Role, Testing, Coding, and Reimbursement.* Chest, 2007. **131**(6): p. 1932-1935.

289. Townley, R.G., et al., *Bronchial sensitivity to methacholine in current and former asthmatic and allergic rhinitis patients and control subjects.* Journal of Allergy and Clinical Immunology, 1975. **56**(6): p. 429-442.

290. Hargreave, F.E., et al., *Bronchial responsiveness to histamine or methacholine in asthma: measurement and clinical significance.* Journal of Allergy and Clinical Immunology, 1981. **68**(5): p. 347-355.

291. Yurdakul, A.S., et al., *The Assessment of Validity of Different Asthma Diagnostic Tools in Adults.* Journal of Asthma, 2005. **42**(10): p. 843 - 846.

292. Weiss, S.T., M.L. Van Natta, and R.S. Zeiger, *Relationship between Increased Airway Responsiveness and Asthma Severity in the Childhood Asthma Management Program.* Am. J. Respir. Crit. Care Med., 2000. **162**(1): p. 50-56.

293. Ryan, G., et al., *Bronchial responsiveness to histamine: relationship to diurnal variation of peak flow rate, improvement after bronchodilator, and airway calibre.* Thorax, 1982. **37**(6): p. 423 - 429.

294. Lewis, S.A., S.T. Weiss, and J.R. Britton, *Airway responsiveness and peak flow variability in the diagnosis of asthma for epidemiological studies.* Eur Respir J, 2001. **18**(6): p. 921-927.

295. Prieto, J.L., et al., *Sensitivity and maximal response to methacholine in prennial and seasonal allergic rhinitis.* Clinical & Experimental Allergy, 1996. **26**(1): p. 61-7.

296. Chinn, S., et al., *Variation in bronchial responsiveness in the European Community Respiratory Health Survey (ECRHS).* Eur Respir J, 1997. **10**(11): p. 2495-2501.

297. Cockcroft, D.W., B.A. Berscheid, and K.Y. Murdock, *Unimodal distribution of bronchial responsiveness to inhaled histamine in a random human population.* Chest, 1983. **83**(5): p. 751-754.

298. Salome, C.M., et al., *Perception of airway narrowing in a general population sample.* Eur Respir J, 1997. **10**(5): p. 1052-1058.

299. Laprise, C., et al., *Asymptomatic airway hyperresponsiveness: relationships with airway inflammation and remodelling.* Eur Respir J, 1999. **14**(1): p. 63-73.

300. Laprise, C. and L.-P. Boulet, *Asymptomatic Airway Hyperresponsiveness: A Three-year Follow-up.* Am. J. Respir. Crit. Care Med., 1997. **156**(2): p. 403-409.

301. Zhong, N.S., et al., *Is asymptomatic bronchial hyperresponsiveness an indication of potential asthma? A two-year follow-up of young students with bronchial hyperresponsiveness.* Chest, 1992. **102**(4): p. 1104-1109.

302. ATS, *Guidelines for Methacholine and Exercise Challenge Testing---1999.* Am. J. Respir. Crit. Care Med., 2000. **161**(1): p. 309-329.

303. Jain, P., et al., *Utility of Peak Expiratory Flow Monitoring.* Chest, 1998. **114**(3): p. 861-876.

304. Paggiaro, P.L., et al., *Relationship between peak expiratory flow (PEF) and FEV_1.* Eur Respir J, 1997. **24**(Suppl): p. 39S - 41S.

305. Llewellin, P., et al., *The relationship between FEV1 and PEF in the assessment of the severity of airways obstruction.* Respirology, 2002. **7**(4): p. 333-337.

306. Aggarwal, A.N., D. Gupta, and S.K. Jindal, *The Relationship Between FEV1 and Peak Expiratory Flow in Patients With Airways Obstruction Is Poor.* Chest, 2006. **130**(5): p. 1454-1461.

307. Hetzel, M.R. and T.J. Clark, *Comparison of normal and asthmatic circadian rhythms in peak expiratory flow rate.* Thorax, 1980. **35**(10): p. 732 - 738.

308. Verschelden, P., et al., *Compliance with and accuracy of daily self-assessment of peak expiratory flows (PEF) in asthmatic subjects over a three month period.* Eur Respir J, 1996. **9**(5): p. 880-885.

309. Enright, P.L., D.L. Sherrill, and M.D. Lebowitz, *Ambulatory Monitoring of Peak Expiratory Flow: Reproducibility and Quality Control.* Chest, 1995. **107**(3): p. 657-661.

310. Holcroft, C.A., et al., *Measurement Characteristics of Peak Expiratory Flow.* Chest, 2003. **124**(2): p. 501-510.

311. Douma, W.R., et al., *Changes in peak expiratory flow indices as a proxy for changes in bronchial hyperresponsiveness. Dutch Chronic Non-Specific Lung Disease study group.* Eur Respir J, 2000. **16**(2): p. 220-225.

312. Neukirch, F., et al., *Peak expiratory flow variability and bronchial responsiveness to methacholine. An epidemiologic study in 117 workers.* American Review of Respiratory Disease, 1992. **146**(1): p. 71 - 75.

313. Goldstein, M.F., et al., *Comparisons of Peak Diurnal Expiratory Flow Variation, Postbronchodilator FEV1 Responses, and Methacholine Inhalation Challenges in the Evaluation of Suspected Asthma.* Chest, 2001. **119**(4): p. 1001-1010.

314. Kerstjens, H.A., et al., *Influence of treatment on peak expiratory flow and its relation to airway hyperresponsiveness and symptoms. The Dutch CNSLD Study Group.* Thorax, 1994. **49**(11): p. 1109 - 1115.

315. Doi, S., et al., *CD4 T-lymphocyte activation is associated with peak expiratory flow variability in childhood asthma.* Journal of Allergy and Clinical Immunology, 1996. **97**(4): p. 955 - 962.

316. Wright, B.M., *A miniature Wright peak-flow meter.* British Medical Journal, 1978. **2**(6152): p. 1627 - 1628.

317. Miller, M.R., P.R. Atkins, and O.F. Pedersen, *Inadequate peak expiratory flow meter characteristics detected by a computerised explosive decompression device.* Thorax, 2003. **58**(5): p. 411-416.

318. Siersted, H.C., et al., *Evaluation of peak expiratory flow variability in an adolescent population sample. The Odense Schoolchild Study.* Am. J. Respir. Crit. Care Med., 1994. **149**(3 Pt 1): p. 598 - 603.

319. Reddel, H.K., et al., *Which index of peak expiratory flow is most useful in the management of stable asthma?* Am. J. Respir. Crit. Care Med., 1995. **151**(5): p. 1320 - 1325.

320. Higgins, B.G., et al., *The distribution of peak expiratory flow variability in a population sample.* American Review of Respiratory Disease, 1989. **140**(5): p. 1368 - 1372.

321. Miles, J.F., et al., *The performance of Mini Wright peak flow meters after prolonged use.* Respiratory Medicine, 1995. **89**(9): p. 603-605.

322. *Workshop summary and guidelines: Investigative use of bronchoscopy, lavage, and bronchial biopsies in asthma and other airway diseases.* Journal of Allergy and Clinical Immunology, 1991. **88**(5): p. 808-814.

323. *British Thoracic Society guidelines on diagnostic flexible bronchoscopy.* Thorax, 2001. **56**(90001): p. i1-21.

324. Busse, W.W., et al., *Investigative Bronchoprovocation and Bronchoscopy in Airway Diseases.* Am. J. Respir. Crit. Care Med., 2005. **172**(7): p. 807-816.

325. Elston, W.J., et al., *Safety of research bronchoscopy, biopsy and bronchoalveolar lavage in asthma.* Eur Respir J, 2004. **24**(3): p. 375-377.

326. Humbert, M., et al., *Safety of fibreoptic bronchoscopy in asthmatic and control subjects and effect on asthma control over two weeks.* Thorax, 1996. **51**(7): p. 664 - 669.

327. Djukanovic, R., et al., *The safety aspects of fibreoptic bronchoscopy, bronchoalveolar lavage, and endobronchial biopsy in asthma.* American Review of Respiratory Disease, 1991. **143**(4 Pt 1): p. 772 - 777.

328. Kurimoto, N., et al., *Assessment of Usefulness of Endobronchial Ultrasonography in Determination of Depth of Tracheobronchial Tumor Invasion.* Chest, 1999. **115**(6): p. 1500-1506.

329. *Osiris Medical Imaging Software*. 2003, University of Geneva.

330. Britten, K.M., P.H. Howarth, and W.R. Roche, *Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies.* Biotechnic and histochemistry: official publication of the Biological Stain Commission, 1993. **68**(5): p. 271 - 80.

331. Nelson, P.N., et al., *Monoclonal antibodies.* Mol Pathol, 2000. **53**(3): p. 111-7.

332. Polak, J.M. and S. van Noorden, *Introduction to Immunosytochemistry*. Second ed. 1997, Oxford UK: BIOS Scientific Publishers Ltd.

333. Jordan, W.J., *Enzyme-Linked Immunosorbent Assay*, in *Medical Biomethods Handbook*, J.M. Walker and R. Rapley, Editors. 2005, Humana Press. p. 419-427.

334. Chomczynski, P. and N. Sacchi, *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.* Analytical Biochemistry, 1987. **162**(1): p. 156-159.

335. Verma, I.M., *Studies on reverse transcriptase of RNA tumor viruses III. Properties of purified Moloney murine leukemia virus DNA polymerase and associated RNase H.* J Virol., 1975. **15**(4): p. 843-854.

336. Mullis, K., et al., *Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. 1986.* Biotechnology, 1992. **24**: p. 17-27.

337. Saiki, R.K., et al., *Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase.* Science, 1988. **239**(4839): p. 487-491.

338. Eckert, K.A. and T.A. Kunkel, *High fidelity DNA synthesis by the Thermus aquaticus DNA polymerase.* Nucleic Acids Res, 1990. **18**(13): p. 3739-3744.

339. Bustin, S.A. and T. Nolan, *Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction.* J Biomol Tech, 2004. **15**(3): p. 155-166.

340. Gelse, K., E. Pöschl, and T. Aigner, *Collagens--structure, function, and biosynthesis.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1531-1546.

341. Shoulders, M.D. and R.T. Raines, *Collagen Structure and Stability.* Annual Review of Biochemistry, 2009. **78**(1): p. 929-958.

342. Canty, E.G. and K.E. Kadler, *Procollagen trafficking, processing and fibrillogenesis.* J Cell Sci, 2005. **118**(7): p. 1341-1353.

343. Banos, C.C., A.H. Thomas, and C.K. Kuo, *Collagen fibrillogenesis in tendon development: Current models and regulation of fibril assembly.* Birth Defects Research Part C: Embryo Today: Reviews, 2008. **84**(3): p. 228-244.

344. Bergeron, C., et al., *Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: Effect of hydrofluoroalkane-flunisolide.* Journal of Allergy and Clinical Immunology, 2005. **116**(5): p. 983-989.

345. Kariyawasam, H.H., et al., *Remodeling and Airway Hyperresponsiveness but Not Cellular Inflammation Persist after Allergen Challenge in Asthma.* Am. J. Respir. Crit. Care Med., 2007. **175**(9): p. 896-904.

346. Bergeron, C., et al., *Regulation of procollagen I (alpha 1) by interleukin-4 in human bronchial fibroblasts: a possible role in airway remodelling in asthma.* Clinical & Experimental Allergy, 2003. **33**(10): p. 1389-1397.

347. Laliberté, R., et al., *Decreased capacity of asthmatic bronchial fibroblasts to degrade collagen.* Matrix Biology, 2001. **19**(8): p. 743-753.

348. Burgess, J.K., *The role of the extracellular matrix and specific growth factors in the regulation of inflammation and remodelling in asthma.* Pharmacology & Therapeutics, 2009. **122**(1): p. 19-29.

349. Vignola, Antonio M., et al., *Sputum Metalloproteinase-9/Tissue Inhibitor of Metalloproteinase-1 Ratio Correlates with Airflow Obstruction in Asthma and Chronic Bronchitis.* Am. J. Respir. Crit. Care Med., 1998. **158**(6): p. 1945-1950.

350. Mautino, G., et al., *Tissue Inhibitor of Metalloproteinase-1 Levels in Bronchoalveolar Lavage Fluid from Asthmatic Subjects.* Am. J. Respir. Crit. Care Med., 1999. **160**(1): p. 324-330.

351. Brown, N.J., et al., *Airway Distensibility in Adults with Asthma and Healthy Adults, Measured by Forced Oscillation Technique.* Am. J. Respir. Crit. Care Med., 2007. **176**(2): p. 129-137.

352. Jeffery, P.K., et al., *Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity.* American Review of Respiratory Disease, 1989. **140**(6): p. 1745 - 1753.

353. McCarter, J.H. and J.J. Vazquez, *The bronchial basement membrane in asthma.* Archives of Pathology, 1966. **82**(4): p. 328 - 335.

354. Pepe, C., et al., *Differences in airway remodeling between subjects with severe and moderate asthma.* Journal of Allergy and Clinical Immunology, 2005. **116**(3): p. 544-549.

355. Chetta, A., et al., *Vascular Remodelling and Angiogenesis in Asthma: Morphological Aspects and Pharmacological Modulation.* Inflammation & Allergy - Drug Targets (Formerly `Current Drug Targets - Inflammation & Allergy), 2007. **6**: p. 41-45.

356. Kuwano, K., et al., *Small airways dimensions in asthma and in chronic obstructive pulmonary disease.* American Review of Respiratory Disease, 1993. **148**(5): p. 1220 - 1225.

357. King, Gregory G., Nestor L. Muller, and Peter D. Pare, *Evaluation of Airways in Obstructive Pulmonary Disease Using High-Resolution Computed Tomography.* Am. J. Respir. Crit. Care Med., 1999. **159**(3): p. 992-1004.

358. Paganin, F., et al., *Chest radiography and high resolution computed tomography of the lungs in asthma.* American Review of Respiratory Disease, 1992. **146**(4): p. 1084 - 1087.

359. Hurter, T. and P. Hanrath, *Endobronchial sonography: feasibility and preliminary results.* Thorax, 1992. **47**(7): p. 565 - 567.

360. Tanaka, F., et al., *Evaluation of tracheo-bronchial wall invasion using transbronchial ultrasonography (TBUS).* European Journal of Cardio-Thoracic Surgery, 2000. **17**(5): p. 570-574.

361. Baba, M., et al., *Correlation between endobronchial ultrasonography (EBUS) images and histologic findings in normal and tumor-invaded bronchial wall.* Lung Cancer, 2002. **35**(1): p. 65-71.

362. Sorkness, R.L., et al., *Lung function in adults with stable but severe asthma: air trapping and incomplete reversal of obstruction with bronchodilation.* J Appl Physiol, 2008. **104**(2): p. 394-403.

363. Yilmaz, S., et al., *High-resolution computed tomography findings in elderly patients with asthma.* European Journal of Radiology, 2006. **59**(2): p. 238-243.

364. Takemura, M., et al., *Bronchial dilatation in asthma: relation to clinical and sputum indices.* Chest, 2004. **125**(4): p. 1352-8.

365. Brackel, H.J.L., et al., *Central Airways Behave More Stiffly during Forced Expiration in Patients with Asthma.* Am. J. Respir. Crit. Care Med., 2000. **162**(3): p. 896-904.

366. Bourdin, A., et al., *Specificity of basement membrane thickening in severe asthma.* Journal of Allergy and Clinical Immunology, 2007. **119**(6): p. 1367-1374.

367. Bernfield, M., et al., *Functions of Cell Surface Heparan Sulfate Proteoglycans.* Annual Review of Biochemistry, 1999. **68**(1): p. 729-777.

368. Kalamajski, S. and Å. Oldberg, *The role of small leucine-rich proteoglycans in collagen fibrillogenesis.* Matrix Biology, 2010. **29**(4): p. 248-253.

369. Prydz, K. and K. Dalen, *Synthesis and sorting of proteoglycans.* J Cell Sci, 2000. **113**(2): p. 193-205.

370. Visse, R. and H. Nagase, *Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases: Structure, Function, and Biochemistry.* Circ Res, 2003. **92**(8): p. 827-839.

371. Westergren-Thorsson, G., et al., *Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects.* The International Journal of Biochemistry & Cell Biology, 2002. **34**(10): p. 1256-1267.

372. Rada, J.A., P.K. Cornuet, and J.R. Hassell, *Regulation of Corneal Collagen Fibrillogenesis In Vitro by Corneal Proteoglycan (Lumican and Decorin) Core Proteins.* Experimental Eye Research, 1993. **56**(6): p. 635-648.

373. Kresse, H. and E. Schönherr, *Proteoglycans of the extracellular matrix and growth control.* Journal of Cellular Physiology, 2001. **189**(3): p. 266-274.

374. Danielson, K.G., et al., *Targeted Disruption of Decorin Leads to Abnormal Collagen Fibril Morphology and Skin Fragility.* J. Cell Biol., 1997. **136**(3): p. 729-743.

375. Scott, J.E., *Proteodermatan and Proteokeratan Sulfate (Decorin, Lumican/Fibromodulin) Proteins Are Horseshoe Shaped. Implications for Their Interactions with Collagen.* Biochemistry, 1996. **35**(27): p. 8795-8799.

376. Border, W.A., et al., *Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease.* Nature, 1992. **360**(6402): p. 361-364.

377. Iozzo, R.V., et al., *Decorin is a biological ligand for the epidermal growth factor receptor.* J Biol Chem, 1999. **274**(8): p. 4489-92.

378. De Luca, A., et al., *Decorin-induced Growth Suppression Is Associated with Up-regulation of p21, an Inhibitor of Cyclin-dependent Kinases.* J. Biol. Chem., 1996. **271**(31): p. 18961-18965.

379. Schönherr, E., et al., *Interaction of Biglycan with Type I Collagen.* J. Biol. Chem., 1995. **270**(6): p. 2776-2783.

380. Ameye, L., et al., *Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis.* FASEB J., 2002. **16**(7): p. 673-680.

381. Hildebrand, A., et al., *Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta.* Biochem J., 1994. **302**(Pt 2): p. 527-34.

382. Ferdous, Z., et al., *Decorin-transforming Growth Factor- Interaction Regulates Matrix Organization and Mechanical Characteristics of Three-dimensional Collagen Matrices.* J. Biol. Chem., 2007. **282**(49): p. 35887-35898.

383. Zhang, G., et al., *Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development.* Journal of Cellular Biochemistry, 2006. **98**(6): p. 1436-1449.

384. Chakravarti, S., *Functions of lumican and fibromodulin: Lessons from knockout mice.* Glycoconjugate Journal, 2002. **19**(4): p. 287-293.

385. Svensson, L., I. Narlid, and A. Oldberg, *Fibromodulin and lumican bind to the same region on collagen type I fibrils.* FEBS letters, 2000. **470**(2): p. 178-182.

386. Yang, B.L., et al., *Cell adhesion and proliferation mediated through the G1 domain of versican.* Journal of Cellular Biochemistry, 1999. **72**(2): p. 210-220.

387. Yang, B.L., et al., *Versican G3 domain enhances cellular adhesion and proliferation of bovne intervertebral disc cells cultured in vitro.* Life Sci., 2003. **73**(26): p. 3399-413.

388. Zhang, Y., et al., *The G3 Domain of Versican Enhances Cell Proliferation via Epidermial Growth Factor-like Motifs.* J. Biol. Chem., 1998. **273**(33): p. 21342-21351.

389. Zhang, Y., et al., *Promotion of chondrocyte proliferation by versican mediated by G1 domain and EGF-like motifs.* Journal of Cellular Biochemistry, 1999. **73**(4): p. 445-457.

390. Merrilees, M.J., et al., *Retrovirally Mediated Overexpression of Versican V3 by Arterial Smooth Muscle Cells Induces Tropoelastin Synthesis and Elastic Fiber Formation In Vitro and In Neointima After Vascular Injury.* Circ Res, 2002. **90**(4): p. 481-487.

391. Wicks, J., et al., *Enhanced upregulation of smooth muscle related transcripts by TGF□2 in asthmatic (myo) fibroblasts.* Thorax, 2006. **61**(4): p. 313-319.

392. Broide, D.H., et al., *Cytokines in symptomatic asthma airways.* Journal of Allergy and Clinical Immunology, 1992. **89**(5): p. 958-967.

393. Virchow, J.C., et al., *T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma.* Am. J. Respir. Crit. Care Med., 1995. **151**(4): p. 960-8.

394. Tillie-Leblond, I., et al., *Balance between Proinflammatory Cytokines and Their Inhibitors in Bronchial Lavage from Patients with Status Asthmaticus.* Am. J. Respir. Crit. Care Med., 1999. **159**(2): p. 487-494.

395. Yamauchi, K., et al., *High levels of transforming growth factor-beta are present in the epithelial lining fluid of the normal human lower respiratory tract.* Am. Rev. Respir. Dis., 1988. **137**(6): p. 1360-3.

396. Redington, A.E., et al., *Transforming Growth Factor-beta 1 in Asthma . Measurement in Bronchoalveolar Lavage Fluid.* Am. J. Respir. Crit. Care Med., 1997. **156**(2): p. 642-647.

397. Zhang, S., et al., *Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma.* Lab. Invest., 1999. **79**(4): p. 395-405.

398. Johnson, P.R.A., et al., *The Production of Extracellular Matrix Proteins by Human Passively Sensitized Airway Smooth-Muscle Cells in Culture . The Effect of Beclomethasone.* Am. J. Respir. Crit. Care Med., 2000. **162**(6): p. 2145-2151.

399. Tufvesson, E. and G. Westergren-Thorsson, *Alteration of proteoglycan synthesis in human lung fibroblasts induced by interleukin-1beta and tumor necrosis factor-alpha.* Journal of Cellular Biochemistry, 2000. **77**(2): p. 298-309.

400. Kahari, V.M., H. Larjava, and J. Uitto, *Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-beta 1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but down-regulates decorin (PGII) mRNA levels in human fibroblasts in culture.* J. Biol. Chem., 1991. **266**(16): p. 10608-10615.

401. Venkatesan, N., P.J. Roughley, and M.S. Ludwig, *Proteoglycan expression in bleomycin lung fibroblasts: role of transforming growth factor-beta 1 and interferon-gamma.* Am J Physiol Lung Cell Mol Physiol, 2002. **283**(4): p. L806-814.

402. Hallgren, O., et al., *Altered fibroblast proteoglycan production in COPD.* Respiratory Research, 2010. **11**(1): p. 55.

403. Liu, F.-T., *Regulatory Roles of Galectins in the Immune Response.* International Archives of Allergy and Immunology, 2005. **136**(4): p. 385-400.

404. Rabinovich, G.A., et al., *An Emerging Role for Galectins in Tuning the Immune Response: Lessons from Experimental Models of Inflammatory Disease, Autoimmunity and Cancer.* Scandinavian Journal of Immunology, 2007. **66**(2-3): p. 143-158.

405. Barondes, S.H., et al., *Galectins. Structure and function of a large family of animal lectins.* J. Biol. Chem., 1994. **269**(33): p. 20807-20810.

406. Cooper, D.N.W., *Galectinomics: finding themes in complexity.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2002. **1572**(2-3): p. 209-231.

407. Rabinovich, G.A., *Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy.* Cell Death and Differentiation, 1999. **6**(8): p. 711-721.

408. Perillo, N.L., M.E. Marcus, and L.G. Baum, *Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death.* Journal of Molecular Medicine, 1998. **76**(6): p. 402-412.

409. Rabinovich, G.A., et al., *Functions of cell surface galectin-glycoprotein lattices.* Current Opinion in Structural Biology, 2007. **17**(5): p. 513-520.

410. Dumic, J., S. Dabelic, and M. Flogel, *Galectin-3: An open-ended story.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2006. **1760**(4): p. 616-635.

411. Hughes, R.C., *Secretion of the galectin family of mammalian carbohydrate-binding proteins.* Biochimica et Biophysica Acta (BBA) - General Subjects, 1999. **1473**(1): p. 172-185.

412. Gong, H.C., et al., *The NH2 Terminus of Galectin-3 Governs Cellular Compartmentalization and Functions in Cancer Cells.* Cancer Res, 1999. **59**(24): p. 6239-6245.

413. Ahmad, N., et al., *Galectin-3 Precipitates as a Pentamer with Synthetic Multivalent Carbohydrates and Forms Heterogeneous Cross-linked Complexes.* J. Biol. Chem., 2004. **279**(12): p. 10841-10847.

414. Liu, F.T., et al., *Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages.* Am J Pathol, 1995. **147**(4): p. 1016-1028.

415. Hughes, R.C., *Mac-2: a versatile galactose-binding protein of mammalian tissues.* Glycobiology, 1994. **4**(1): p. 5-12.

416. Gritzmacher, C.A., M.W. Robertson, and F.T. Liu, *IgE-binding protein. Subcellular location and gene expression in many murine tissues and cells.* J Immunol, 1988. **141**(8): p. 2801-2806.

417. Moutsatsos, I.K., et al., *Endogenous Lectins from Cultured Cells: Nuclear Localization of Carbohydrate-Binding Protein 35 in Proliferating 3T3 Fibroblasts.* PNAS, 1987. **84**(18): p. 6452-6456.

418. Liu, F.-T., R.J. Patterson, and J.L. Wang, *Intracellular functions of galectins.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2002. **1572**(2-3): p. 263-273.

419. Frigeri, L.G. and F.T. Liu, *Surface expression of functional IgE binding protein, an endogenous lectin, on mast cells and macrophages.* J Immunol, 1992. **148**(3): p. 861-867.

420. Sato, S. and R.C. Hughes, *Regulation of secretion and surface expression of Mac-2, a galactoside- binding protein of macrophages.* J. Biol. Chem., 1994. **269**(6): p. 4424-4430.

421. Yang, R.-Y., D.K. Hsu, and F.-T. Liu, *Expression of galectin-3 modulates T-cell growth and apoptosis.* PNAS, 1996. **93**(13): p. 6737-6742.

422. Akahani, S., et al., *Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family.* Cancer Res, 1997. **57**(23): p. 5272-5276.

423. Matarrese, P., Ornella Fusco Nicola Tinari Clara Natoli Fu-Tong Liu Maria Letizia Semeraro Walter Malorni Stefano Iacobelli, *Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties.* International Journal of Cancer, 2000. **85**(4): p. 545-554.

424. Nakahara, S., N. Oka, and A. Raz, *On the role of galectin-3 in cancer apoptosis.* Apoptosis, 2005. **10**(2): p. 267-275.

425. Shimonishi, T., et al., *Expression of endogenous galectin-1 and galectin-3 in intrahepatic cholangiocarcinoma.* Human Pathology, 2001. **32**(3): p. 302-310.

426. Sanjuan, X., et al., *Differential expression of galectin 3 and galectin 1 in colorectal cancer progression.* Gastroenterology, 1997. **113**(6): p. 1906-1915.

427. Inohara, H. and A. Raz, *Functional evidence that cell surface galectin-3 mediates homotypic cell adhesion.* Cancer Res, 1995. **55**(15): p. 3267-3271.

428. Inohara, H., et al., *Interactions between galectin-3 and Mac-2-binding protein mediate cell-cell adhesion.* Cancer Res, 1996. **56**(19): p. 4530-4534.

429. Kuwabara, I. and F.T. Liu, *Galectin-3 promotes adhesion of human neutrophils to laminin.* J Immunol, 1996. **156**(10): p. 3939-3944.

430. Hsu, D.K. and F.-T. Liu, *Regulation of cellular homeostasis by galectins.* Glycoconjugate Journal, 2002. **19**(7 - 9): p. 507-515.

431. Acosta-Rodriguez, E.V., et al., *Galectin-3 Mediates IL-4-Induced Survival and Differentiation of B Cells: Functional Cross-Talk and Implications during Trypanosoma cruzi Infection.* J Immunol, 2004. **172**(1): p. 493-502.

432. Joo, H.-G., et al., *Expression and function of galectin-3, a β-galactoside-binding protein in activated T lymphocytes.* J Leukoc Biol, 2001. **69**(4): p. 555-564.

433. Sato, S., et al., *Role of Galectin-3 as an Adhesion Molecule for Neutrophil Extravasation During Streptococcal Pneumonia.* J Immunol, 2002. **168**(4): p. 1813-1822.

434. Craig, S., Krishnaswamy, P, Irani, AM, Kepley, CL, Liu, FT, Schwartz, LB., *Immunoelectron microscopic localization of galectin-3, an IgE binding protein, in human mast cells and basophils.* The Anatomical record., 1995. **242**(2): p. 211-9.

435. Sano, H., et al., *Human Galectin-3 Is a Novel Chemoattractant for Monocytes and Macrophages.* J Immunol, 2000. **165**(4): p. 2156-2164.

436. Sano, H., et al., *Critical role of galectin-3 in phagocytosis by macrophages.* J. Clin. Invest., 2003. **112**(3): p. 389-397.

437. Yamaoka, A., et al., *A human lectin, galectin-3 (epsilon bp/Mac-2), stimulates superoxide production by neutrophils.* J Immunol, 1995. **154**(7): p. 3479-3487.

438. Papaspyridonos, M., et al., *Galectin-3 Is an Amplifier of Inflammation in Atherosclerotic Plaque Progression Through Macrophage Activation And Monocyte Chemoattraction.* Arterioscler Thromb Vasc Biol, 2007: p. ATVBAHA.107.159160.

439. Henderson, N.C., et al., *Galectin-3 regulates myofibroblast activation and hepatic fibrosis.* Proceedings of the National Academy of Sciences, 2006. **103**(13): p. 5060-5065.

440. Wang, L., et al., *Galectin-1 and galectin-3 in chronic pancreatitis.* Laboratory Investigation, 2000. **80**(8): p. 1233 - 1241.

441. Sharma, U.C., et al., *Galectin-3 Marks Activated Macrophages in Failure-Prone Hypertrophied Hearts and Contributes to Cardiac Dysfunction.* Circulation, 2004. **110**(19): p. 3121-3128.

442. Lippert, E., et al., *Soluble galectin-3 is a strong, colonic epithelial-cell-derived, lamina propria fibroblast-stimulating factor.* Gut, 2007. **56**(1): p. 43-51.

443. Nishi, Y., et al., *Role of galectin-3 in human pulmonary fibrosis.* Allergol Int, 2007. **56**(1): p. 57-65.

444. Pilette, C., et al., *Increased galectin-3 expression and intra-epithelial neutrophils in small airways in severe COPD.* Eur Respir J, 2007. **29**(5): p. 914-922.

445. Zuberi, R.I., et al., *Critical Role for Galectin-3 in Airway Inflammation and Bronchial Hyperresponsiveness in a Murine Model of Asthma.* Am J Pathol, 2004. **165**(6): p. 2045-2053.

446. Mengwasser, J., F.T. Liu, and J.P. Sleeman, *Galectin-3 is strongly up-regulated in nonapoptosing mammary epithelial cells during rat mammary gland involution.* Glycobiology, 2002. **12**(2): p. 129-34.

447. Minshall, E., et al., *IL-11 expression is increased in severe asthma: Association with epithelial cells and eosinophils.* The Journal of Allergy and Clinical Immunology, 2000. **105**(2): p. 232-238.

448. Araujo, B.B., et al., *Extracellular matrix components and regulators in the airway smooth muscle in asthma.* Eur Respir J, 2008. **32**(1): p. 61-69.

449. Johnson, P.R.A., et al., *Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism.* The Journal of Allergy and Clinical Immunology, 2004. **113**(4): p. 690-696.

450. Hirst, S.J., C.H.C. Twort, and T.H. Lee, *Differential Effects of Extracellular Matrix Proteins on Human Airway Smooth Muscle Cell Proliferation and Phenotype.* Am. J. Respir. Cell Mol. Biol., 2000. **23**(3): p. 335-344.

451. Castagnaro, A., et al., *High-resolution computed tomography evaluation of airway distensibility in asthmatic and healthy subjects.* Radiol Med, 2008. **113**(1): p. 43-55.

452. Vonk, J.M., et al., *Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up.* Thorax, 2003. **58**(4): p. 322-327.

453. Ohno, I., et al., *Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation.* Am J Respir Cell Mol Biol, 1996. **15**(3): p. 404-9.

454. Vignola, A.M., et al., *Transforming Growth Factor-beta Expression in Mucosal Biopsies in Asthma and Chronic Bronchitis.* Am. J. Respir. Crit. Care Med., 1997. **156**(2): p. 591-599.

455. Magnan, A., et al., *Altered compartmentalization of transforming growth factor-beta in asthmatic airways.* Clinical & Experimental Allergy, 1997. **27**(4): p. 389-95.

456. Balzar, S., et al., *Increased TGF-β2 in severe asthma with eosinophilia.* The Journal of Allergy and Clinical Immunology, 2005. **115**(1): p. 110-117.

457. Ludwig, M.S., et al., *Mechanical strain enhances proteoglycan message in fibroblasts from asthmatic subjects.* Clinical & Experimental Allergy, 2004. **34**(6): p. 926-930.

458. Bellego, F.L., et al., *Mechanical strain increases cytokine and chemokine production in bronchial fibroblasts from asthmatic patients.* Allergy, 2009. **64**(1): p. 32-39.

459. Potter-Perigo, S., et al., *Regulation of Proteoglycan Synthesis by Leukotriene D4 and Epidermal Growth Factor in Bronchial Smooth Muscle Cells.* Am. J. Respir. Cell Mol. Biol., 2004. **30**(1): p. 101-108.

460. Todorova, L., et al., *Budesonide/formoterol effects on metalloproteolytic balance in TGFβ-activated human lung fibroblasts.* Respiratory Medicine, 2009. **103**(11): p. 1755-1763.

461. Kasper, M. and R.C. Hughes, *Immunocytochemical evidence for a modulation of galectin 3 (Mac-2), a carbohydrate binding protein, in pulmonary fibrosis.* The Journal of Pathology, 1996. **179**(3): p. 309-316.

462. Ge, X.N., et al., *Allergen-Induced Airway Remodeling Is Impaired in Galectin-3-Deficient Mice.* J Immunol, 2010. **185**(2): p. 1205-1214.

463. Bates, J.H.T., M. Rincon, and C.G. Irvin, *Animal models of asthma.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(3): p. L401-410.

464. Lensmar, C., et al., *Phenotypic analysis of alveolar macrophages and lymphocytes following allergen inhalation by atopic subjects with mild asthma.* Respiratory Medicine, 2006. **100**(5): p. 918-925.

465. Phipps, S., et al., *Acute Allergen-Induced Airway Remodeling in Atopic Asthma.* Am. J. Respir. Cell Mol. Biol., 2004. **31**(6): p. 626-632.

466. Kelly, M.M., et al., *Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma.* The Journal of Allergy and Clinical Immunology, 2010. **125**(2): p. 349-356.e13.

467. Sziksz, E., et al., *Galectin-9 in Allergic Airway Inflammation and Hyper-Responsiveness in Mice.* International Archives of Allergy and Immunology, 2010. **151**(4): p. 308-317.

468. Devouassoux, G., et al., *Galectin-10 mRNA is overexpressed in peripheral blood of aspirin-induced asthma.* Allergy, 2008. **63**(1): p. 125-131.

469. Perillo, N.L., et al., *Apoptosis of T cells mediated by galectin-1.* Nature, 1995. **378**(6558): p. 736-9.