Structure Function Relationships in Adult Asthma.

Thesis submitted for the degree of Doctor of Philosophy
At the University Of Leicester (2008)
by

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Abstract.

Airway hyperresponsiveness (AHR) is a central feature of the asthma paradigm. Non Asthmatic Eosinophilic Bronchits (EB) has emerged as a powerful disease control model to study the immunopathological mechanisms of AHR in asthma. EB is characterised by cough, AHR is absent despite ongoing eosinophilic airway inflammation. This thesis compares features of structural and cellular remodeling in the airway wall in asthma and EB as well as the static geometry of proximal conducting airways in these two polar conditions. We have demonstrated for the first time that structural remodeling, notably increased airway smooth muscle (ASM) mass, vascular remodeling and the expression of VEGF, number of submucosal glands and collagen 3 deposition; occur to similar degrees in both asthma and EB. None of these structural components were associated with AHR in asthma. In contrast we found that the number of mast cells within the ASM independently correlated with the degree of AHR. Coupled with these findings, asthma was characterised by reduced patency of the proximal airway lumen due to airway wall thickening and the degree of thickening correlated with the degree of AHR. In contrast EB was characterised by maintained proximal airway luminal patency despite an increase in the area of the airway wall. Our findings suggest that AHR is dissociated from airway wall structural remodeling in asthma and associated with mast cell infiltration of the ASM. Finally we have shown that fibrocytes are present in the ASM in asthma in contrast to EB and may contribute to the increased ASM mass seen in asthma. Future studies should explore the mechanisms that promote reduced luminal patency in asthma and preserve luminal patency in EB, as well as the functional impact of mast cell infiltration of the ASM and fibrocytes in asthma upon ASM dynamics.

Acknowledgements

I would like to thank the patients and volunteers that participated in the studies and made this work possible.
I would like to thank my supervisor Chris Brightling who made my research possible by securing funding for me from Asthma UK and for all of his support and supervision over the last 3 years. Chris has also encouraged me to develop as an independent researcher. Peter Bradding has offered advice and reviewed all of the studies that I have performed and his help as an additional mentor has been invaluable.
Vijay Mistry and Camille Doe have provided invaluable technical support with immunohistochemistry. Will Monteiro and Amanda Sutcliffe have been pivotal in the laboratory with sputum processing and analysis of sputum supernatants respectively.
A key aspect of the clinical studies in this thesis has been the careful characterisation of patients for which I would like to thank Sue McKenna in particular, Beverley Hargadon, Hilary Pateman and Maria Shelley.
I would like to thank my colleagues Sumit Gupta and Glenn Cruse for their help in the assessment of airway geometry repeatability and CT analysis. I would also like to thank Pranab Haldar for his help with characterisation and recruitment of patients with refractory asthma.
I would like to thank Dr Sarah Hainsworth, a material science engineer at Leicester University, with her help in the validation of airway measurements using CT and construction of a phantom airway model. I would also like to thank Sam McDonald and Professor Philip Whither for their help and access to Micro CT at the Manchester Material Sciences Centre. Harvey Coxson aided us greatly with advice and access to the Emphylyx-J software that was used to analyse airway wall geometry.
I would like to thank Martyn Foster and Sarah Bolton at Astra Zeneca for access to microscopy equipment that aided thresholding analysis for airway matrix.
I would like to acknowledge Asthma UK and GSK for funding my research.
I dedicate this thesis to my father, Mohammed Hameedullah Siddiqui, who struggled hard to get me a grammar school place many years ago and dedicated his working life to the education of underprivileged minorities in Birmingham. I owe a great debt to my

mother Najma who supported me and believed in me before others and has guided me throughout the years.
Finally special thanks go to my wife Uzma who has supported me through these 3 long and at times arduous PhD years and helped me through the highs and lows of long commutes to Leicester. Our son Ismael was born towards the end of my PhD and cheered me up no end.

Publications and prizes arising from this thesis

1 Papers

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4 Prizes

The British Thoracic Society Young Investigator of the Year 2008 (Runner up)

The West Midlands Respiratory Glaxo Smith Kline Prize 2008
Best spoken SPR presentation

The Wendy Stannard Prize 2007
Best Research Presentation, Trent Research Day.

Commended Oral Presentation: University of Leicester: Department of Infection Immunity and Inflammation 2007.

Commended Oral Presentation: University of Leicester: Department of Infection Immunity and Inflammation 2006

International Society for Hypertension in Pregnancy
Travel Award 2006

4 Abstracts

As a result of this thesis 16 abstracts have been submitted to the British Thoracic Society, American Thoracic Society, European Respiratory Society, British Society for Allergy and Clinical Immunology, Royal College of Radiology and The International Society for Hypertension in Pregnancy.

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1. Introduction

1.1 Definitions and Epidemiology of Asthma.

1.1.1 Asthma: A historical perspective

The term 'Asthma' was first defined by Hippocrates in 460 B.C and was used to define a group of individuals that would pant or breath hard. A more advanced observation of the modern syndrome of asthma was later provided by the ancient greek physician Aretaeus of Cappadocia in A.D 150, ''*They go into the open air, since no house suffices for their respiration; they breathe standing, as if desiring to draw in all the air they can possibly inhale; and, in their want of air, they also open the mouth as if best to enjoy more of it. Pale in countenance, except the cheeks, which are ruddy; sweat about the forehead and clavicles; cough incessant and laborious; expectoration small, thin, cold resembling the efflorescence of foam; neck swells with the inflation of the breath*[1]". Early definitions of asthma therefore defined the disease in terms of component symptoms such as breathlessness, cough and wheeze. The first systematic definition of asthma recognising the disease as an airway disease characterised by variable airflow limitation distinct from cardiovascular disease was proposed in 1958 where asthma was described as ''*a condition of subjects with widespread narrowing of the bronchial airways, which changes its severity over short periods of time, either spontaneously or under treatment and is not due to a cardiovascular disease*[2]''. With the advent of bronchoscopic airway sampling in the 1980's the concept of airway inflammation evolved [3] and the definition of asthma was refined to include a description of airway inflammation. In 1995 the Global Initiative for Asthma (GINA) developed a systematic description of asthma and defined it as '*'a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread but variable airflow*

obstruction that is often reversible, either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli''[4]. The GINA definition identifies the importance of airway hyper responsiveness (AHR) and airway inflammation in the asthma process. This thesis will examine how structural changes in the conducting airways in asthma, denoted by the term 'remodeling' may lead to airway hyper responsiveness and airflow limitation in asthma.

1.1.2 Asthma as a multidimensional disease

One of the problems with defining asthma using single domains such as variable airflow limitation, AHR, symptoms or airway inflammation is that in any individual patient symptoms, inflammation, variable airflow limitation and AHR may be dissociated. For example some asthmatics may have frequent exacerbations with few symptoms and other may be highly symptomatic and have little demonstrable airway inflammation and rarely exacerbate. The importance of identifying discordance between symptoms and inflammation in asthma has been highlighted by a recent asthma phenotypes study that demonstrated that inflammation guided management of asthma in patients with discordant symptoms led to a reduction in exacerbations in patients with eosinophilic airway inflammation (EAI) and few symptoms and a reduction in treatment in patients with symptom predominance without EAI[5].

1.1.3 Epidemiology and healthcare economic impact of asthma

The incidence of asthma has been rising over the last four decades. It is estimated that 300 million people suffer from asthma worldwide and that there has been an overall increase in the prevalence of asthma by 50% per decade. Furthermore the number of hospital admissions due to asthma has increased particularly in young children. Although asthma deaths have been falling since the 1980s, approximately 180,000 patients worldwide die from asthma annually; these deaths predominantly occur in patients in their mid forties and are largely preventable. It is estimated that the western world spends 300-1300 dollars /patient/year on asthma and a large proportion of this resource is used to treat patients with asthma that is difficult to control[6].

1.1.4 Severe asthma, an important and unmet healthcare need

The majority of patients with asthma have mild to moderate disease that can be controlled adequately with inhaled corticosteroids combined with bronchodilators. However subsets of patients have disease that is difficult to control despite high doses of inhaled corticosteroids and other add on therapy. This may be the case despite extensive specialist re-evaluation of the patients disease expression and management. These patients have been branded with a variety of labels in the medical literature, 'difficult asthma, brittle asthma and steroid dependant asthma[7]'. In 1999 the European respiratory society defined the term 'difficult' asthma[8] and subsequently the American Thoracic Society defined the term 'refractory asthma[9]'. The Global Initiative for asthma (GINA) guidelines have also defined severity largely based upon treatment required to achieve adequate asthma control[10]. All of these definitions are broadly based upon the degree of asthma control, response to asthma therapy and the number of exacerbations. More recently an ERS workshop statement on severe asthma has defined the disease as being reserved to those patients who have refractory asthma despite extensive re evaluation over a 6 month period[11].

Severe asthma is an important healthcare challenge and 5-10% of the total asthma population are estimated to continue to have poor control despite combination treatment with high dose inhaled corticosteroids and bronchodilators. This small polar group of individuals account for 50% of the asthma healthcare budget due to recurrent hospital admissions and unscheduled physician visits[12].

This thesis will examine the relationship between airway structure and function in mild to moderate and severe refractory asthma.

1.2 Airway remodeling in asthma.

1.2.1 Definition and limitations of conventional studies

Airway remodeling in asthma was first described in fatal asthma by Huber and Koessler in 1922[13]. Remodeling of the airway in asthma involves an alteration in the size, mass or number of tissue structural components that occurs during growth or in response to inflammation or injury[14]. The working definition of remodeling implies that some

aspects of remodeling are a normal response of the developing airway, whereas other aspects may be aberrant and related to cellular inflammation and injury of the airway wall.

Perhaps the major defining problem of the remodeling process in disease is the fact that the majority of studies that have assessed remodeling in asthma have been cross sectional and thus have not quantified the relationship between remodeling with time and duration of disease.

Most of the longitudinal studies of remodeling have been epidemiological assessments of the relationship of factors such as wheeze, atopy and disordered lung function early in life with lung function decline and adult asthma[15-19]. These studies are limited by the fact that FEV_1 may be a poor surrogate for the remodeling process.

Furthermore few studies have attempted to assess the impact of remodeling of different airway compartments upon the micromechanical properties of the airway wall, such studies have by enlarge been limited to mathematical models[20;21] and studies assessing the relationship between airway compliance/distensibility and structural remodeling [22-24].

Progress in the study of remodeling will require a more detailed assessment of how different components of the remodeling process affects the micromechanics of the airway and ultimately affect the macro mechanics of airway function commonly measured by conventional pulmonary function (FEV_1 and PC_{20}). Furthermore less conventional and evolving measures of airway function such as measures of airway distensibility[25] and resistance using forced oscillation, measures of modelled airflow using computational fluid dynamics in segmented airways derived from imaging modalities such as CT[26;27] and Helium-MRI[28;29], may provide a more detailed insight into the relationship between structure and function in asthma.

1.2.2 Tools used to measure airway remodeling.

A host of clinical tools have been used to assess structural and cellular remodeling *in vivo*. The majority of modern pathological remodeling studies have used endobronchial biopsies (EBB) due to the relative ease of performing the procedure and minimal complication rates compared with transbronchial lung biopsy and open lung biopsy.

Furthermore techniques and research aspects of endobronchial biopsies have been outlined and standardised[30]. One major limitation of EBB is sampling bias due to partial sampling of the airway wall.

A number of studies have also utilised distal lung sampling with transbronchial biopsy. However there remains a significant risk of major complications such as bleeding and pneumothorax and poor success rate (30-50%)[30]. Furthermore a number of biopsies are needed to counteract the poor success rate. Balzar et al have demonstrated that cell density and pattern of inflammatory cell distribution was comparable to surgical lung biopsies in transbronchial biopsies in severe asthma[31].

A number of postmortem studies have assessed remodeling in fatal and non fatal asthma[32-43]. Post mortem studies have allowed a detailed assessment of the entire airway wall in both large and small airways as well as assessment of the bronchial and pulmonary arteries. However one clear limitation of these studies is the influence of mechanical ventilation, hospital admission and associated escalation of treatment upon airway pathology.

Lung function testing has been used by a variety of childhood[17-19;44-46] and adult[47-49] epidemiological studies to assess the relationship with disordered lung function in childhood and the risk of adult asthma, as well as the factors that predict excessive FEV_1 decline and airflow limitation in adult asthma. Another proxy measure of remodeling is reduced airway distensibility defined by the change in anatomical dead space with lung volume[50;51] or measured using the forced oscillation technique[25]. Indeed one study has demonstrated an inverse correlation of RBM thickening with airway distensibility in asthma[24].

Induced sputum and bronchalveolar lavage (BAL) have also been used to indirectly assess cellular remodeling and fluid phase mediators that may be related to the remodeling process. The most powerful evidence for induced sputum lies in its ability to detect eosinophilic airway inflammation (EAI) and targeting EAI has been shown to predict response to inhaled and oral corticosteroids[52-54] and to reduce exacerbations[55-57].

A variety of fluid phase mediators have been measured in BAL and sputum supernatant including vascular endothelial growth factor (VEGF)[58;59]as a proxy for angiogenesis) and the ratio of matrix metalloproteinase to their tissue inhibitors

(eg:MMP9/TIMP1[60;61]as a proxy for matrix turnover). Sputum VEGF was shown to correlate with vascular permeability of the airway in asthma[62] and MMP9/TIMP1 ratios have been shown to relate to airway wall thickening assessed by computed tomography (CT) in asthma[63]. Perhaps one of the major limitations of BAL is the inability to standardise fluid recovery and separate the relative alveolar and distal airway contribution. Similarly induced sputum processing requires the addition of a mucolytic agent which can interfere with immunoassays and reduce the concentration of cytokines and necessitates the need for dialysis of sputum prior to cytokine measurement[64]. In contrast to BAL however, induced sputum is relatively easy, non invasive and can be performed before or after airway challenge as well as in children and in patients during exacerbations.

More recently multi detector computed tomography (MDCT) has been used to assess global airway wall thickening in proximal conducting airways[65-68] and recent data using three dimensional segmentation algorithms has demonstrated that airway remodeling at the 6th airway generation correlates better with post bronchodilator lung function in COPD than at the 3rd generation[69]. Air trapping and areas of low attenuation have also been shown quantitatively to correlate with airflow limitation in asthma[70], however pathological studies defining the airway structural defect that leads to low attenuation in asthma are lacking unlike studies correlating pathological emphysema with area of low attenuation on CT [71]. Unfortunately there are few studies that have compared airway pathology using endobronchial biopsy with airway wall thickening on CT. Furthermore CT is limited by its inability to differentiate structural components in the airway wall and the lack of resolving power to assess small airway geometry.

Endobronchial ultrasound (EBUS) of the airway via a flexible bronchoscope has been used to delineate thickening of different airway wall compartments[72] in a transplant study of obliterative bronchiolitis, the repeatability of compartmental measures using EBUS was good across all 5 identified airway wall compartments. Another study has demonstrated that wall thickening identified using EBUS correlated well with CT wall thickening[73]. However EBUS is limited by the inability to correlate thickening with appropriate structural change in the airway wall eg: ASM mass, matrix etc.

More recently tissue engineered models of the airway wall have been developed to study remodeling[74;75].

1.2.3 Structural remodeling in adult asthma.

A number of clinical studies have attempted to define structural remodeling in asthma. These studies have all been cross sectional and have involved endobronchial[76-80]/transbronchial biopsy tissue[31;81;82] or tissue obtained at post mortem[32-42]. However they have all demonstrated largely consistent findings in proximal conducting airways; notably damage and shedding of the airway epithelium[76;83-87], increased smooth muscle mass[32;76;79;88], a reduction in the distance of the smooth muscle from the airway epithelium [76;79], increased vascularity[89;90], thickening of the reticular basement membrane[76;80;91;92], glandular hyperplasia[36;76]; with conflicting data regarding sub epithelial deposition of airway matrix with some studies shown increased matrix deposition in asthma[76;93] and others showing no difference compared to healthy controls[94;95]. Airway wall thickening of proximal conducting airways as marker of global wall remodeling has been demonstrated in a number of computed tomography studies of asthma[63;96].

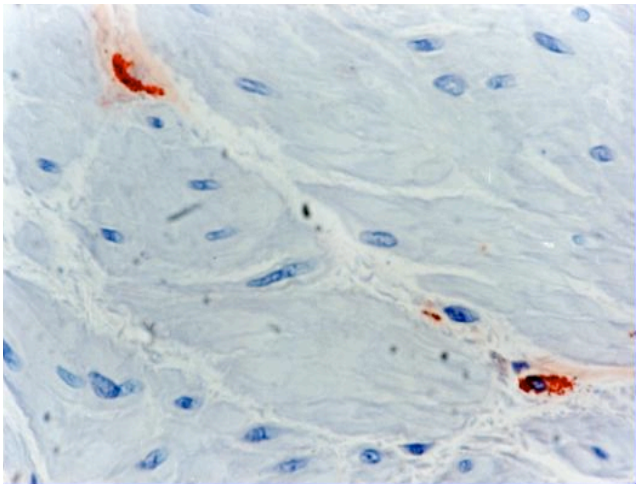
These features of remodeling are present to different extents in other airway diseases including COPD (reviewed in[14]), cystic fibrosis[97;98] and non-C.F bronchiectasis[99].

1.2.4 Cellular remodeling *in vivo* in adult asthma

The pattern of cellular infiltration of the airway wall in asthma is characterised by an increase of eosinophils, mast cells, CD4+ T cells, macrophages; as well as neutrophils and CD8+ T cells during exacerbations and fatal attacks respectively (reviewed in[14]). Perhaps the most consistent finding with regards to cellular infiltration of the airway wall in asthma is the presence of eosinophilic airway inflammation in sputum, BAL, bronchial wash and endobronchial biopsies [100]. However despite the consistent association of eosinophils with corticosteroid response and exacerbations (reviewed in[101]), clinical studies of anti-IL-5 have failed to demonstrate an impact upon important asthma outcomes to date [102-104] and have been limited by the lack of studies powered to

assess a reduction in exacerbations in patients with eosinophilic airway inflammation and recurrent exacerbations, who are most likely to respond to this therapeutic target. Intuitively for inflammatory cells to have an important functional effect upon structural cells in the airway, close proximity within a few microns is likely to be required for direct cellular communication. This concept has been enforced by the observation that mast cell infiltration of the airway smooth muscle in asthma is a key feature of the asthma phenotype and relates to the degree of AHR in asthma[105] (Figure 1.1). However there is a general lack of research into cellular co localisation to structural cells in the airway wall in asthma. The most repeatable findings to date are the localisation of eosinophils and T-cells to the airway epithelium and mast cells to the proximal airway smooth muscle in asthma (reviewed in[106]). Further research is needed to establish how these patterns of cellular localisation may lead to disordered airway physiology in asthma and to examine the functional importance of cellular localisation to other structural components of the airway wall.

Figure: 1.1 Mast cell localisation to the ASM bundle in asthma



Photomicrograph of mast cells (stained red) in an ASM-bundle from a bronchial biopsy taken from an asthmatic (x400).

1.2.5 Is the pattern of airway remodeling consistent between the proximal and distal airways?

The small airways are typically defined as those less than 2 mm in internal diameter. The majority of studies that have assessed features of both small and large airway remodeling have been limited to post mortem studies and a small number of combined endobronchial and transbronchial biopsy studies. Current CT technology does not have the resolving power to assess airway wall remodeling of small airways.

Balzar et al examined cell numbers in large and small airway samples taken using endobronchial, transbronchial and surgical biopsies respectively and failed to find any differences in lymphocytes, granulocytes or mast cells within the lamina propria. In contrast cell density (a composite of all cells) in the lamina propria was found to be increased in small airways compared to large and medium conducting airways. In this study a small airway within transbronchial biopsy specimens was defined as an airway without cartilage and or mucous glands and had alveolar tissue attached to it. Small airways in surgical biopsy tissue were defined as membranous airways with a basement membrane perimeter of <6mm in the absence of cartilage and or mucous glands[31].

Carroll et al assessed cell numbers in large and small airways using post mortem tissue from patients with fatal and non fatal asthma and healthy controls. Eosinophil and lymphocyte numbers were found to be increased in the small membranous (basement membrane perimeter < 6mm) airways in fatal asthmatics compared to healthy controls and in both fatal and non fatal asthmatics in the small cartilaginous airways (basement membrane perimeter 6-16mm) compared to controls. However no differences were found between eosinophil numbers in membranous airways between non fatal asthmatics and healthy controls. Lymphocyte and eosinophil density within the airway wall increased with increasing airway size and eosinophils counts/density were greater in cartilaginous airways compared to membranous airways in fatal asthma but not in non fatal asthmatics or controls[34].

Hamid et al have also demonstrated increased numbers of activated eosinophils in the small airways of asthmatics (internal perimeter < 2mm) compared to larger airways (internal perimeter > 2mm). Both eosinophils and T cells were increased in the small

airways compared to healthy subjects. However these results may have been confounded by concurrent smoking in study subjects[81].

Kraft et al studied patients with nocturnal asthma and non nocturnal asthma and took endobronchial and transbronchial biopsies at 4am and 4pm. Proximal airway eosinophils and CD3+/CD4+/CD8+ T cells were increased at both 4am and 4pm in the lamina propria of proximal compared to small conducting airways. In contrast no differences were observed between proximal and distal airways in cellular localisation to the epithelium[107].

Haley et al examined the distribution of eosinophils within the inner (between smooth muscle and alveolar attachments) and outer wall (between the basement membrane and smooth muscle) of both large and small airways in asthma. Eosinophil and CD45 + cell numbers were significantly increased in the inner airway wall in the large airways in asthma compared to the small airways where eosinophil density was greatest in the outer airway wall[108].

Hauber et al demonstrated that the number of eosinophils, IL-5 and eotaxin + cells were similar in the proximal and small airways prior to treatment with HFA-flunisolide. suggesting that the observed differences in cellular localisation between large and small airways in other studies may be related to inhaled corticosterioid treatment with large particle inhalers causing preferential deposition in the proximal airway wall[82].

In a similar study Minshall et al demonstrated that IL-5 and IL-4 mRNA expression was increased in the airway wall in both large and small airways compared to healthy subjects. However IL-5 expression was increased in the small airways compared to the large airways. No Differences were seen in IL-2 or IFN-γ mRNA expression between large and small airways[109].

Therefore a consistent finding of biopsy studies is an increase in the number of eosinophils and lymphocytes in the small airways in asthma compared to the small airways in healthy subjects. In contrast there is conflicting data with regards to inflammatory cell influx in large vs. small airways which may be due to differences in treatment, bias in assessment between the outer and inner airway wall and diurnal variation in inflammatory cell influx to the airways in asthma.

Few studies have examined selective cellular localisation to structural cells in the large and small airways. However mast cell infiltration of the airway smooth muscle has been consistently observed in proximal airway smooth muscle and has also been reported in small airway wall smooth muscle in asthma[43;77;105;110-113] and mast cell density was increased in membranous compared to cartilaginous airways, in the epithelium and ASM in one study[43].

Structural remodeling of the airway wall may occur in both proximal and small airways in asthma. Hamid et al demonstrated that collagen 3 deposition and ASM mass were similar in both proximal and small airways. In contrast total collagen deposition was greater in proximal airways. 6 weeks treatment with HFA-Flunisolide resulted in a reduction in ASM mass in the small airways only with no impact upon collagen/collagen 3 deposition in the proximal or small airways[114]. This study was limited by the absence of a healthy control group.
Saetta at al assessed autopsy tissue in 6 asthmatics that had died of fatal asthma and controls for structural changes in small airways and adjacent pulmonary arteries. Smooth muscle thickness, the degree of luminal occlusion and eosinophilic infiltration the airway wall was increased in the small airways in asthma. Interestingly there were no structural differences in the intima or media of adjacent pulmonary arteries[115].
Ebina et al demonstrated that increased ASM mass was feature of both large and small airways in asthma[116]. Aikawa et al have demonstrated that the % of mucus glands, goblet cells and the mucus occupying ratio was increased in both central and peripheral airways in fatal asthma[117]. In contrast Hashimoto et al failed to demonstrate any increase in percentage vascularity in the inner or outer airway wall in asthmatics/healthy subjects between large and small airways[89]. James et al demonstrated that the RBM thickness in large cartilaginous airways correlated with both inner airway wall area and ASM mass in small cartilaginous but not small membranous airways, suggesting that thickening of the RBM in proximal airways may be a useful proxy of global remodeling in smaller cartilaginous but not membranous airways[118]. Finally Carroll et al demonstrated that thickening of the inner, outer and total airway wall was feature of

both large and small airways in fatal and non fatal asthmatics compared to healthy subjects[32].

Therefore structural remodeling notably increased ASM mass, vascular remodeling, collagen 3 deposition in the subepithelium and glandular hyperplasia as well as thickening of the inner and outer airway wall are features of both proximal and small conducting airways in asthma.

1.2.6

Selective recruitment of inflammatory cells to airway structural cells

Inflammatory cells microlocalise to specific compartments within the airway with an abundance of some and a paucity of other cells within airway structures. This raises the question: what are the mechanisms controlling the selective recruitment of inflammatory cells to structural compartments within the airway?

Granulocyte trafficking has been extensively studied and characterised[119]. For example the recruitment of eosinophils into the airway is mediated by a multistep process directed by TH_2 cytokines. The first step is increased production and release of eosinophils from the bone marrow under the influence of IL-5[120;121] and specific chemoattractants such as CCL11(eotaxin)[122], CCL5(RANTES) [123], CCL12(MCP-5) [123] and CCL3(MIP-1α)[123]. Secondary target organ vasculature has increased adhesiveness for eosinophils through the specific effects of locally generated IL-4 and IL-13. These cytokines induce the expression of VCAM-1, which binds to eosinophils through the VLA-4 receptor which is not expressed by neutrophils and P-Selectin to which eosinophils bind with greater avidity than neutrophils[124-126]. The interaction of the eosinophil with these adhesion molecules is mediated by integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$)[127;128] which have been shown to bind to VCAM-1[129]. Following adhesion transmigration across the endothelium is mediated by complement proteins such as C5a[130]. The final phase of trafficking involves chemotaxis, which is mediated by chemokines such as CCL11[131] and involves encryption and passage through the matrix network of the airway wall. CCL11 has been shown to bind to CCR3, a high affinity receptor expressed by eosinophils and mediates directional migration through tissue[132]. Survival of eosinophils is augmented by IL-5[133] and GM-CSF[134]. In contrast the mechanisms

involved in the recruitment of inflammatory cells in tissue to structural components are poorly understood.

Selective recruitment of inflammatory cells to the ASM is likely to be mediated by smooth muscle derived chemoattractants and by the maintenance of the correct microenvironment to maintain cell differentiation and survival. ASM has a significant secretory capacity, so it clearly has the potential to recruit inflammatory cells[135]. For example, CXCL8 (IL-8) and CXCL10 (IP-10) released by activated ASM in COPD may mediate neutrophil and CD8+ cell migration into the ASM-bundle in the small airway[136;137]. A plethora of chemotactic factors for mast cells are released by ASM notably stem cell factor (SCF)[110], CCL11 (eotaxin)[138-140], CXCL8 (IL-8)[140;141], CX_3CR1[111] and transforming growth factor (TGF)-β [110]. CXCR3 is the most abundantly expressed chemokine receptor on human lung mast cells within the ASM-bundle. Human lung mast cell migration is induced by the CXCR3 ligand CXCL10, which is released preferentially from Th_1-stimulated asthmatic ASM cells compared to those from healthy controls[142] and is released in response to TLR3 activation[143]. Interestingly, Th_2-stimulated ASM from asthmatics is chemotactic for mast cells compared to non-asthmatic ASM mediated via activation of CCR3 and CXCR1. This is not due to differential expression of chemokines, but is likely to be due to the release of a factor that inhibits mast cell migration released by the non-asthmatic ASM[140]. It is also important to consider why some inflammatory cells notably eosinophils and T-cells are rarely seen in the ASM in spite of appropriate chemotactic signals. Eosinophil paucity in ASM may be explained by selective cleavage of eosinophil chemoattractants CCL5 (RANTES) and CCL11 (eotaxin) by mast cell ß-tryptase[144]. Similar interactions may explain the lack of other cells such as T-lymphocytes from ASM bundles in asthma as Lazaar et al demonstrated that mast cell chymase inhibited integrin mediated T-cell adhesion to ASM cells[145].

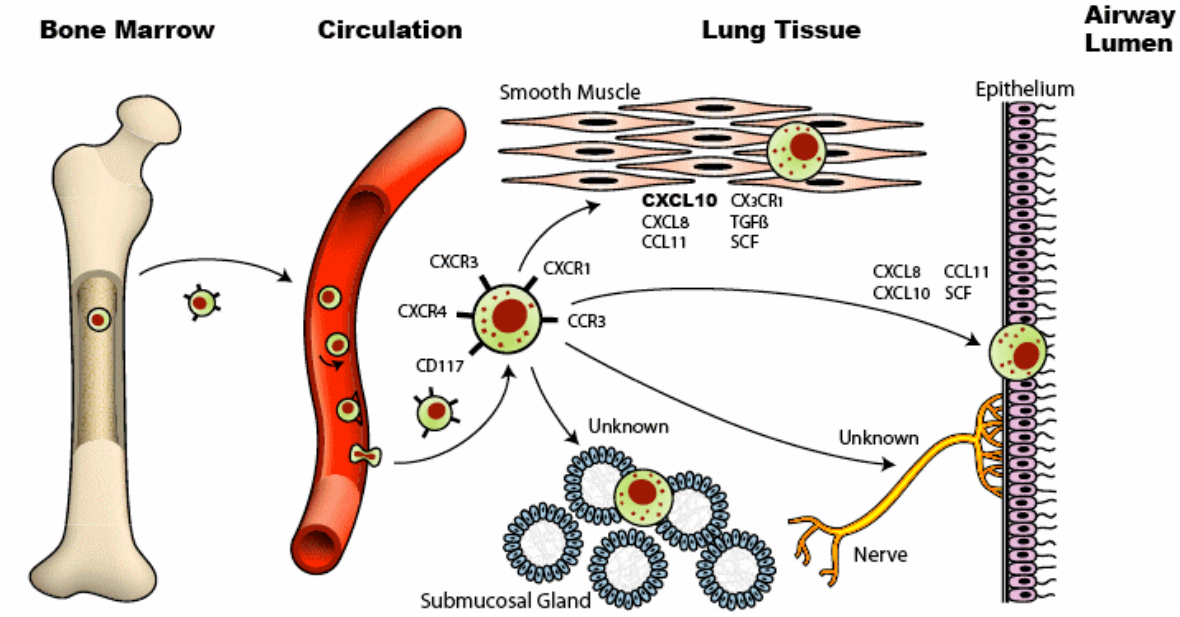
Similarly, the airway epithelium has the capacity to secrete a number of chemokines that are involved in the recruitment of inflammatory cells[146;147]. The induced sputum CXCL10 concentration from subjects with non-asthmatic eosinophilic bronchitis is increased and mediates mast cell migration via CXCR3 activation[148]. Airway

epithelium is an important source of CCR3 ligands, which play a role in the recruitment of mast cells, eosinophils and Th_2 cells. CXCL8 is an important neutrophil and mast cell chemoattractant and its expression is increased in induced sputum from subjects with COPD[149;150] and neutrophilic asthma[151], and is increased in response to cigarette smoke[152]. How chemokines interact to recruit inflammatory cells to the airway epithelium is poorly understood and why there is less selection in cell recruitment than observed in the ASM is unknown.

Chemokine expression by airway mucus glands has not been extensively examined. CXCL8 mRNA expression by mucus glands is increased in response to pseudomonas infection in bronchiectasis[153]. However, to date there is no data to explain the selective recruitment of mast cells and neutrophils to the airway mucus glands in asthma.
The mechanisms described to date involved in the selective microlocalisation of mast cells to ASM, epithelium and mucosal glands in asthma is summarised in (Figure 1.2). Eosinophil localisation to airway nerves is mediated by release of CCR3 ligands by nerves[154]. It would be predicted that airway nerve-derived CCL11 would also recruit Th_2 cells and mast cells, but whether eosinophil localisation to the airway nerves in human disease is selective is unclear.

It is likely that a number of chemokines, other chemotaxins and inhibitory factors play a role in the selective recruitment of inflammatory cells into the airway. As described above it may be that the release of chemotaxins by ASM and other airway structural cells varies in response to different stimuli such as cigarette smoke, infection or allergen exposure. Future studies should explore the relative importance of these triggers and the associated network of chemotaxins that are released in promoting selective inflammatory cell microlocalisation to structural compartments of the airway.

Figure 1.2: Mast cell trafficking to airway structures



In the airway mast cells traffic to airway structures namely the ASM-bundle, epithelium and mucus glands. The mechanisms known to be involved in this microlocalisation are as shown.

1.2.7 Mechanisms of structural remodeling in asthma.

The most repeatable structural changes within the proximal airway wall in asthmatics compared to healthy subjects are increased ASM mass, RBM thickening, glandular hyperplasia/mucus secretion and vascular remodeling (reviewed in[14]).

An increase in ASM mass in asthmatics compared to healthy subjects has been reported in a number of biopsy studies of remodeling. Furthermore in severe asthma the distance of the ASM from the airway epithelium is reduced. The increase in observed ASM mass is likely to be due to a combination of hypertrophy and hyperplasia[76;88;116] as well as the deposition of extracellular matrix around ASM cells[155]. It is likely that the local inflammatory micro environment facilitates both hyperplasia and hypertrophy. IL-13 a cardinal TH_2 cytokine has been shown to augment CysLT1 receptor expression on human ASM *in vitro* and augments proliferation by CysLTs[156]. Similarly a number of mediators including TGF-β[157] and endothelin-1[158] have been shown to promote ASM hypertrophy. The hyperplasia of ASM muscle cells may result from the differentiation and migration of mesenchymal cells beneath the airway epithelium acting in synergy with the epithelium as an epithelial-mesenchymal trophic unit[159]. Thickening of the reticular basement membrane has also been reported in most adult asthma biopsy studies. This feature of the remodeling process seems to develop early in life as demonstrated by a recent paediatric bronchscopy study that demonstrated thickening of the RBM in 'confirmed wheezers' between the age of 1 and 3 years compared to reported wheezy children and non wheezers[160]. In this study confirmed wheeze was associated with a greater degree of eosinophilic infiltration in the submucosa compared to reported wheezy children and non wheezers; suggesting that thickening of the RBM may be related to EAI. The link between EAI and RBM thickening is strengthened by a recent biopsy study comparing eosinophilic vs. non eosinophilic asthmatics, defined by the presence or absence of EAI in serial induced sputum samples. RBM thickening was increased in EA compared to healthy subjects but not in NEA[161]. Furthermore in animal models of allergic asthma, mice with complete and selective ablation of the eosinophil lineage fail to develop RBM thickening[162].

Vascular remodeling and the increased expression of VEGF a key angiogenic growth factor in induced sputum, BAL and within the airway wall are well recognised features

of the asthmatic airway (reviewed in[163]). The presence of VEGF immunoreactive cells is directly related to the number of submucosal vessels in asthma[164] and increased VEGF in sputum is associated with increased microvascular permeability[59]. Chetta et al demonstrated that the number of mast cells in the sub-epithelium correlates with the number of vessels in asthma[165], however a similar correlation was not observed for eosinophilic airway inflammation. Although studies have described an association with sputum VEGF and % eosinophilia in sputum[59], a similar relationship *in vivo* has not been described. Despite the lack of data linking airway inflammation and vascular remodeling in asthma; growth factors (VEGF, b-FGF and angiogenin) co-localise predominantly to eosinophils, macrophages and CD34+ cells *in vivo* and the expression of these growth factors correlates with the % vascularity of the airway wall[166]. Furthermore the expression of the main signalling receptor for VEGF (FLT-1) is increased in vessels in asthmatics[167]. Taken together these observations suggest that eosinophils, macrophages and mesenchymal cells may be important in regulating vascular remodeling in asthma. However, it remains unclear whether inflammatory cells are selectively co-localised to vessels in asthma.

Mucus production is a cardinal feature of asthma and COPD and mucus gland hyperplasia is a feature of both conditions[117;168-170]. Patients with asthma across the spectrum of severity, including fatal asthma demonstrate increased numbers of degranulated mast cells[43;112] and neutrophils[36] within submucosal glands compared to controls. However the degree of mucus related obstruction expressed as the % of the airway lumen occupied by mucus only correlated with mast cell numbers and not with neutrophil numbers[36]. Mast cell proteases, tryptase and chymase, are potent stimuli for mucus secretion and other mast cell mediators, PGD_2 and LTC_4[171], together with mast cell derived-cytokines IL-6, IL-13[172] and IL-4[173] have also been implicated in glandular hyperplasia and mucus production. More recently, mast cell expression of amphiregullin, a member of the epidermal growth factor family, was increased in asthma and this upregulates mucin gene expression by epithelial cells implicating amphiregullin in goblet cell metaplasia and mucus hypersecretion[174;175]. Similarly, neutrophils may promote mucus hypersecretion by upregulation of MUC5AC by neutrophil elastase[176;177].

1.2.8 Structural remodeling of the ASM in asthma: A consequence of fibrocyte recruitment?

An alternative mechanism for the increase in ASM mass seen in asthma is that mesenchymal progenitor cells may be recruited from the bone marrow to the airway and differentiate into ASM cells[178].

In 1994, a distinct population of blood-borne fibroblast-like cells that rapidly enter sites of tissue injury were described[179] and termed fibrocytes. These cells comprise 0.1–0.5% of non-erythrocytic cells in peripheral blood and display an adherent, spindle-shaped morphology when cultured *in vitro*. Once in tissue it is possible that these fibrocytes differentiate into myofibroblasts and/or smooth muscle cells. In support of this view the number of fibrocyte-like cells were increased in bronchial biopsies from asthmatics[180] and fibrocytes in culture have been shown to express α-smooth muscle actin and contract collagen gels[181]. Myofibroblasts have been described in the airway wall particularly following allergen challenge. It has been hypothesized that these cells are dedifferentiated smooth muscle cells that have migrated towards the epithelium (reviewed in[14]). However, they may also represent a population of cells that have differentiated from resident fibroblasts or possibly migrating fibrocytes.

Possible mechanisms regulating smooth muscle cell or myofibroblast migration in asthma are poorly understood. The best described mechanism for induction of vascular and airway smooth muscle cell migration is ligand-induced activation of receptor tyrosine kinases by platelet-derived growth factor (PDGF) [182]. Chemotactic responses to PDGF were potentiated by cysteinyl-leukotrienes[183] and urokinase[184], although neither were chemotactic themselves. We have recently shown that the CCR7-CCL19 axis may be an important mechanism of ASM hyperplasia in asthma[185].

Wang et al have recently demonstrated that there are increased numbers of circulating fibrocytes in asthmatics with airflow obstruction compared to asthmatics without airflow limitation or healthy controls. In this study the increase in fibrocyte numbers correlated with FEV_1 decline. Furthermore the release of TGF-β was higher in fibrocytes from asthmatics with airflow obstruction and anti TGF-β inhibited

myofibroblastic differentiation of fibrocytes[186]. Therefore fibrocytes may be important in the immunopathogenesis of remodeling.

1.2.9 Can remodeling be modulated by treatment?

Much of the work assessing the impact of treatment upon structural change has focused on inhaled and oral corticosteroids, however novel and emerging therapeutic options include bronchial thermoplasty and a variety of immunomodulatory treatments that have to date been limited to animal models. Furthermore most studies assessing the impact of treatment have focused upon proximal conducting airways and have utilized endobronchial biopsies and CT as outcome measures of remodeling and much of the work assessing the impact of treatment upon cellular remodeling in biopsy studies has failed to focus upon cellular localisation to airway structural components.

A further limitation in the assessment of the impact of treatment upon structural change is the lack of evidence associating compartmental remodeling with disturbed airway function. Tissue turnover and restructuring is a normal homeostatic process[187] and may help to preserve the micromechanical properties of the airway.

Inhaled corticosteroids have been shown to modulate epithelial restoration with partial restoration observed in one retrospective study over a 10-year period[188]; however this was not associated with a change in AHR. 3 months of budesonide therapy has been shown to increase ciliated epithelial cells compared to bronchodilator alone in one prospective study[189]. De Klujiver et al observed a decrease in goblet cell numbers in mild persistent asthma after 2 weeks of treatment with inhaled corticosteroids[190]. Therefore Inhaled corticosteroids seem to modulate epithelial repair in asthma and may have an impact upon mucus production.

Several studies have assessed the impact of inhaled corticosteroids upon RBM thickening and have demonstrated conflicting results with some demonstrating a reduction in thickness after treatment[191-193] and others showing no impact[194;195]. One possible reason for the conflicting evidence may be that studies that have shown reductions in basement membrane thickness suggest that both prolonged and high dose inhaled corticosteroid therapy is necessary and RBM thickening is related to the degree

of EAI which was not measured in many of these studies. Treatment with Mepolizumab a humanized monoclonal antibody to IL-5 resulted in a significant reduction in the expression of tenascin, lumican and procollagen 3 in the reticular basement membrane in asthma accompanied by a 50% reduction in tissue and bone marrow eosinophilia[196].

Modulation of vascular remodeling by inhaled corticosteroids is well established. Hoshino et al demonstrated that 6 months of treatment with 800mcg of BDP daily reduced submuscosal vascularity and basement membrane collagen 3 deposition in asthma and that this reduction was associated with an improvement in FEV_1 and AHR[197]. Furthermore corticosteroids have been shown to reduce induced sputum VEGF one of the key regulators of angiogenesis in asthma and this reduction was associated with an improvement in FEV_1[59]. More recently Feltis et al have demonstrated that 3 months of treatment with inhaled corticosteroids reduced VEGF+ vessels in the airway wall in mild asthma accompanied by a reduction in angiogenic sprouts and VEGF receptor expression[167]. Finally inhaled corticosteroids and leukotriene receptor antagonists have been shown to reduce airway mucosal blood flow to an equivalent degree in asthma[198].

Inhaled corticosteroids have wide ranging effects upon ASM cells including modulation of chemokines and cytokines involved in ASM function and proliferation[199], arrest of ASM in the G1 phase of the cell cycle[200], reduction of contractile protein expression[201]. More recently Roth et al have demonstrated that absence of the CCAAT/ enhancer binding protein α, which forms a complex with the glucocorticoid receptor activating the cell cycle inhibitor P21 may explain the inability of corticosteroids to modulate smooth muscle proliferation in asthmatic ASM cells *ex vivo*[202].
Modulating ASM in proximal conducting airways by radiofrequency ablation of the ASM using thermoplasty has been shown to improve asthma symptoms, peak flow and reduce mild exacerbations[203].
Few studies have assessed the impact of treatment upon matrix deposition in the subepithelium. De Kluijver et al demonstrated that 2 weeks of treatment with inhaled corticosteroids after low dose allergen challenge increased the submucosal density of

the proteoglycans biglycan and versican[190]. In contrast Hamid et al demonstrated that 6 weeks of treatment with HFA-flunisolide had no effect upon large or small airway total collagen or collagen 3 deposition[114].

In asthma oral and inhaled corticosteroids consistently reduce the number of eosinophils in the airway epithelium[189;204]and some, but not all, report reductions in T-cells and mast cells[204]. For other anti-inflammatory therapy the picture is also unclear and the data sparse. There are no reports on the effects of anti-leukotriene therapy on cellular localisation to airway structures, anti-IgE in asthma led to a reduction in T-cells in the airway epithelium[205]. There is therefore a pressing need to understand the effect of current treatment on modulating inflammatory cell localisation to structural cells.

Recruitment of inflammatory cells into the airway is an important target for treatment of asthma. In addition to treatments with broad anti-inflammatory actions a number of antibody and small molecule therapies have been developed or are in development to target specific aspects of cell trafficking[206]. For example, anti-IL-5 reduces the number of eosinophils in the airway[207], but whether this has an impact on eosinophil infiltration into the epithelium is unknown and more importantly whether anti-IL-5 has clinical benefits for example in reducing asthma exacerbations is uncertain. In animal models of asthma CCR3 antagonists reduce the clustering of eosinophils along cholinergic nerves and AHR secondary to M2R dysfunction[154.] Alternative strategies that target events in cellular activation involved in migration may offer novel therapies. For example, the mast cell calcium-activated potassium channel (K(Ca)3.1) modulates the retraction of the cell body during migration. Specific inhibition of this ion channel by TRAM-34 attenuated CXCL10-mediated mast cell migration[208].

Whether inflammatory cell localisation to structural cells can be modulated by current and future treatments needs to be further examined and the functional consequences of this inhibition need to be fully determined.

Studies assessing the impact of evolving immunomodulatory drugs upon structural and cellular remodeling to structural cells, including anti TNF, anti-IgE, blockade of TH_2

cytokines (IL-4, IL-5, IL-13), PDE_4 inhibitors and matrix metalloproteinase inhibitors are eagerly awaited (reviewed in[209]).

Summary (1.2.1-1.2.9)

Airway remodeling in asthma encompasses a variety of structural and cellular changes within the airway wall. Few studies have assessed the impact of cellular localisation to structural cells within the airway wall upon function and the majority of studies have focused on proximal conducting airways.

1.3 Airway inflammation in asthma.

1.3.1 Induced sputum as a marker of granulocytic inflammation.

The ability to obtain an induced sputum sample using hypertonic saline[210] has been a major advance in airways disease. Sputum induction is a well-tolerated, safe and repeatable procedure even in patients with severe disease[211;212]. Induced sputum inflammatory cell counts in asthma correlate well with bronchial wash[213] and reasonably with bronchoalveolar cell counts[213;214]. In contrast the relationship between induced sputum findings and cell counts in bronchial biopsy is more variable[215], possibly due to the fact that granulocytes are not tissue resident cells. In keeping with this observation the suppressive effects of corticosteroid therapy and anti-IL-5 in asthma are more apparent in sputum compared to endobronchial biopsies [104 196].

1.3.2 Eosinophilic airway inflammation and response to treatment.

In asthma sputum eosinophilia has also been shown to correlate with response to corticosteroids[52.] Little et al demonstrated that a sputum eosinophilia of >4% had a positive predictive value of 68% for predicting a > 15 % FEV_1 response to a 2 week oral corticosteroid trial[53]. Furthermore a sputum eosinophilia correlates positively with the degree of improvement to inhaled and oral corticosteroids and seems to be more

closely associated with clinical response than ENO or sputum/peripheral blood eosinophilic cationic protein[52].

A variety of corticosteroid reduction studies have shown that induced sputum eosinophilia precedes asthma exacerbations[216;217], suggesting that strategies targeting sputum eosinophilia can effectively reduce exacerbations. 3 clinical studies have compared symptom and guideline based asthma management to a sputum eosinophil based strategy and have demonstrated that targeting sputum eosinophilia can successfully reduce exacerbations compared to guideline based approaches[55-57.]

1.3.3 Non-eosinophilic asthma: A distinct clinico-pathological disease entity.

Non-eosinophilic asthma (NEA) is defined by clinical symptoms of asthma and AHR in the absence of sputum eosinophilia[218;219]. Values for the upper limit of the normal sputum eosinophil count have varied between studies, Green *et al* used a cut off of < 1.9%[220] whereas Gibson et al used a cut off of < 2.5%[151] and Pavord et al < 3%[221] for the classification of NEA. The 90th centile of the sputum eosinophil count in a healthy population has been reported as 1.8%[222] and 1.9%[223] in 2 large community studies.

NEA extends across the spectrum of asthma severity and the phenotype is unlikely to be simply related to corticosteroid treatment[80;151;221;224].Corticosteroids appear to have limited efficacy in NEA[221.] A recent double blind placebo controlled crossover trial of inhaled mometasone 400µg OD in EA vs. NEA, demonstrated that patients with EA had a significant 5.5 doubling dose improvement in methacholine PC_{20} after 8 weeks of mometasone compared to placebo vs. a 0.5 doubling dose improvement in patients with NEA. Furthermore in the EA group there was a net 1.0 improvement in the Juniper asthma quality of life score compared to placebo vs. a 0.2 improvement in the NEA group ($P<0.05$)[161]. Asthmatic smokers have been shown to have reduced eosinophils and increased neutrophils and IL-8 in sputum compared to asthmatic non smokers[225], features similar to those observed in NEA. However the majority of studies that have assessed patients with NEA have excluded cigarette smokers with a > 10 pack year history and there is no difference in the proportion of ex smokers or never smokers

between NEA and EA in most studies[151;224;226]. Few studies have examined the stability of NEA as an asthma phenotype. Furthermore there are no studies that have examined whether the phenotype is stable during asthma exacerbations, however a subgroup of patients do not generate a sputum eosinophilia during asthma exacerbation[226]. Anees et al assessed the short term reproducibility of NEA in occupational asthma and collected duplicate sputum samples after 1 week and reported no change in asthma classification[227]. A further study examined reproducibility of NEA, utilising 2 sputum samples over a 6-week period, the Kappa statistic (95% CI) was 0.64 (0.4-0.88) indicating moderate agreement in classification between visits[228]. In the same study 7 NEA patients were followed up over a mean of 5.3 yrs, 6/7 remained Non-eosinophilic, indicating substantial long-term reproducibility (Kappa 0.77[0.57-0.97]). In view of the poor response to inhaled corticosteroids in NEA a recent study examined the response to macrolide therapy[229] in patients with refractory asthma and demonstrated that subjects with non eosinophilic asthma had a significant reduction in sputum IL-8, neutrophil numbers and improved quality of life compared to placebo. However one of the defining problems of NEA in corticosteroid treated patients is the inability to distinguish between the effects of treatment upon eosinophilic airway inflammation or a true non eosinophilic phenotype. Furthermore the greatest benefit in the above study was seen in patients with neutrophilic rather than paucigranulocytic inflammation.

Therefore non-eosinophilic asthma represents a reproducible asthma phenotype across the entire spectrum of asthma severity and does not seem to 'respond' to inhaled corticosteroids.

1.3.4 Remodeling in Eosinophilic vs. Non eosinophilic asthma.

A recent endobronchial biopsies study[161] in corticosteroid naïve asthmatics revealed that patients with EA had increased submuscosal tissue eosinophilia and thicker reticular basement membranes, compared to patients with NEA. Interestingly the number of mast cells within the airway smooth muscle did not differ between the two groups but was significantly greater than in matched healthy controls, suggesting that mast cell smooth muscle infiltration in asthma is independent of eosinophilic airway inflammation.

Wenzel et al demonstrated in severe asthma, that non eosinophilic disease demonstrated by an absence of tissue eosinophilia was associated with normal thickness of the reticular basement membrane and a lower number of CD3+ cells and macrophages when compared to patients with eosinophilic inflammation in tissue[80].

Summary(1.3.1-1.3.4)

The use of induced sputum to identify eosinophilc airway inflammation has evolved as powerful biomarker to predict the response to inhaled corticosteroids and exacerbations in asthma. A subgroup of patients with asthma do not have EAI and may have non eosinophilic asthma or neutrophil driven disease. Remodeling of the airway wall in asthma may differ according to the pattern of airway inflammation assessed by induced sputum, indeed NEA is characterised by an absence of RBM thickening, however mast cell infiltration of the ASM is a feature of both EA and NEA.

1.4 Airway hyper responsiveness in asthma: A defining feature of the asthma paradigm.

1.4.1 Assessment of airway hyperresponsiveness in clinical practice

AHR is a fundamental feature of the asthma paradigm and is characterised by the capacity of the airway to narrow in response to a host of direct and indirect stimuli to the airway smooth muscle (ASM). Direct challenge agents such as histamine and methacholine act via receptors located on ASM involved in the regulation of static airway calibre. Indirect agents such as cold air[230], adenosine[231], mannitol[232] and exercise[233] induce bronchoconstriction by the release of mediators from resident airway cells. One of the first clinical descriptions of airway hyperresponsivenes (AHR) was provided by Curry et al[234], who demonstrated that nebulised, intramuscular and intravenous histamine could cause a significant reduction in vital capacity in patients with symptomatic asthma. AHR has since emerged as powerful tool in the management of asthma and to define the asthma phenotype in both clinical and epidemiological studies.

The most commonly available direct challenge agents in clinical practice are methacholine and histamine.

Change in FEV_1 is the primary outcome measure for methacholine challenge testing. The provocation concentration to cause a 20% fall in FEV_1 from baseline or post diluent (PC_{20}) is commonly used to summarise the results.

The PC_{20} can be derived by plotting the change in FEV_1 as percentage of the baseline or post diluent value against the $\log_{10}$ methacholine concentration using the equation below[235;236].

$$\mathbf{PC_{20} = \text{antilog}\ \log C_1 + \frac{(\log C_2 - \log C_1)(20 - R_1)}{(R_2 - R_1)}}$$

where

C_1= second to last methacholine concentration

C_2= final concentration of methacholine causing a >/= 20% fall in FEV_1

R_1= percent fall in FEV_1 after C_1

R_2= percent fall in FEV_1 after C_2

However a number of studies have demonstrated that non-linear models are better at deriving PC_{20} values between doubling doses of methacholine than simple linear models[237;238].

Challenge results are often defined categorically. AHR is considered present when the PC_{20} to histamine or methacholine is <8 mg/ml. However, AHR to methacholine and histamine is unimodally and log normally distributed and the continuity of this normal distribution leads to diagnostic uncertainty when trying to define a 'normal response'.

PC_{20} values of >16 mg/ml make it highly unlikely that a patient has asthma even if the pre test clinical probability is relatively high[239;240]. In contrast if the PC_{20} is <1 mg/ml the diagnosis of asthma is highly likely even with a relatively low pre test clinical probability; in this setting the specificity and positive predictive value of asthma approach 100%[241] in contrast to a relatively poor sensitivity[240]. However in patients with intermediate PC_{20} values between 1-16 mg/ml there is much more diagnostic

uncertainty. For example the positive predictive value of a histamine PC_{20}<8mg/ml for current asthma symptoms is <50%[240]. Therefore perhaps the best clinical utility of methacholine/histamine challenge is its negative predictive value at >16mg/ml. The only caveats to this rule are patients who develop transient AHR in response to allergen exposure[242]or occupational sensitisers[243] and are removed from exposure at the time of challenge testing. Further diagnostic confusion arises in the presence of fixed airflow limitation. Patients with COPD also demonstrate AHR to histamine and methacholine[244-247]. Therefore direct challenge bronchoprovocation lacks the specificity to detect asthma in the presence of airflow limitation.

The repeatability over the short-term for methacholine challenge is ±1.5 doubling doses[248-257]. Recent evidence dose not support the notion that tachyphylaxis occurs to methacholine if challenge is repeated within 24h and repeatability within a single day was within 1 doubling dose[258].

1.4.2 Airway hyperresponsiveness: Mechanisms

1.4.2.1 An innate defect of airway smooth muscle.

The view that ASM is altered in asthma and that it is a critical determinant in the development of AHR is both longstanding and contentious. Early, attempts to prove that the ASM from asthmatics is hypercontractile have met with little success, in that the opportunities to conduct contractile studies on asthma derived muscle are rare and the results of the few studies that do exist vary from demonstrating increased or decreased contractility, decreased relaxation or no clear differences[259-262].

More recently, Ma et al demonstrated that ASM freshly dissociated from bronchial biopsies had both increased velocity of contraction and maximal contraction[263]. Differences in rate of ASM shortening are likely to be important as this is implicated in counteracting the relaxing effect of deep inspiration[264;265], and contributes to the greater extent of maximal airway narrowing[266-268]. ASM has been shown to exhibit length adaptation with recovery of contractile force as muscle length changes; which is likely to be related to adaptability of the contractile filaments and cytoskeleton and helps to maintain optimum contractile protein overlap[269;270] and orientation[271;272]. The adaptation

of contractile filaments is likely to involve changes in both the number of filaments in series and in parallel. The plasticity of contractile filaments is exhibited at the macro scale by the entire smooth muscle cell; which is in a constant state of structural fluidity and adaptation in response to non-thermal agitation of the cytoskeleton, such that the cell behaves like soft glass[273]. Loss of adaptation may be fundamental to aberrant ASM function in asthma. The reversal of the bronchodilatation to deep inspiration would establish a condition in which the ASM remains continually shortened [274;275], raising the possibility of contractile filament rearrangement and muscle length adaptation at shorter lengths[276]. In cultured ASM bundles from rabbits chronic shortening of the bundle was found to be associated with increased passive stiffness and partial loss of the ability of the muscle to adapt when returned to its *in situ* length[277]. Indeed, these phenomena may provide an explanation for the enhanced airway reactivity of the Fisher inbred rat strain, whereby increased cytoskeletal remodeling was observed in cultured ASM from Fisher compared with Lewis rat strains[278]. There is therefore emerging evidence to support the view that ASM contractility is altered in asthma. For these changes in ASM mechanics to occur in asthma it is likely that there are differences in the fundamental mechanisms controlling ASM contraction.

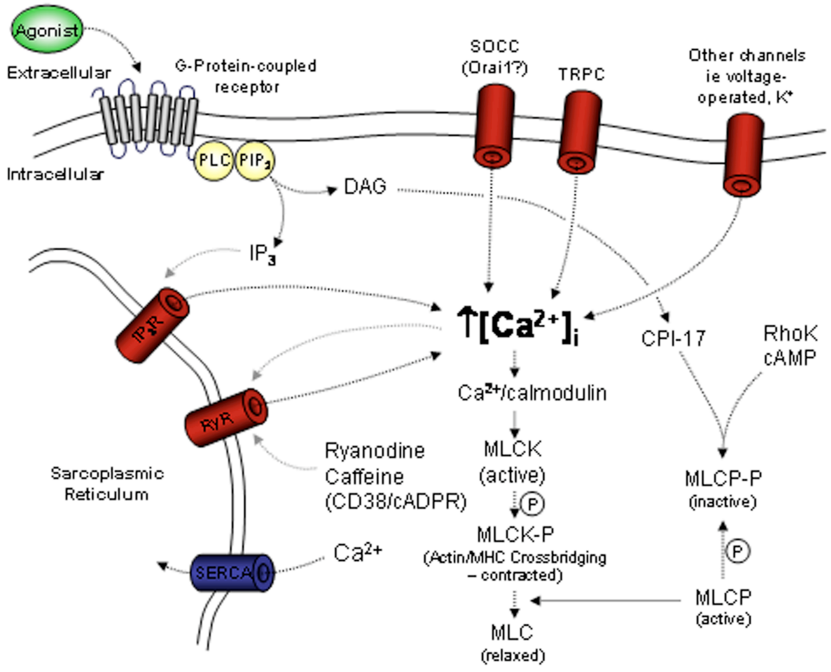
Contraction of ASM is dependent upon activation of actin and myosin cross-bridges and the phosphorylation of myosin light chain by myosin light chain kinase. This process is controlled by the interaction between calcium-excitation coupling and calcium sensitivity[279]. The mechanisms involved in this process are summarised in (figure 1.3). Recent studies suggest that the calcium homeostasis of ASM from asthmatics may be abnormal with increased cytosolic calcium and increased spontaneous calcium oscillations in asthmatic ASM[280;281]. The mechanisms driving this aberrant Ca^{2+} signalling are under investigation and may reveal fundamental differences between ASM in health and disease. A similar process is evident in the Fisher rat strain in which calcium mobilisation is upregulated in response to increased IP3 expression and altered PKC regulation[282]. In human disease, calcium sensitivity is also heightened in ASM with increased expression of myosin light chain kinase reported in freshly dispersed primary cells[263] and in ASM-bundles in bronchial biopsies[76]. This increase in calcium

sensitivity together with an alteration in the control of intracellular calcium would be predicted to contribute to the abnormal ASM mechanics observed in asthma.

Therefore the body of evidence is now swinging in favour of the view that the phenotype of ASM in asthma is fundamentally altered. Changes in calcium homeostasis and calcium sensitivity in ASM from asthmatics provide an explanation for the increased velocity and maximal contraction. This in turns modifies ASM mechanics with ASM-shortening, exaggerated airway narrowing and inhibition of bronchial dilatation in response to deep inspiration. Importantly, these mechanisms do not occur in isolation but are compounded by airway inflammation and remodeling.

In addition to its altered contractile phenotype ASM from asthmatics exhibits changes in its secretory and proliferative capacity[283]. ASM in asthma may also be intrinsically resistant to the anti-proliferative effects of inhaled corticosteroids due to an absence of C/EBP alpha a complex of the glucocorticoid receptor enhancer binding protein[202]. In concert these changes are also likely to contribute to the development of airway remodeling and AHR via recruitment of inflammatory cells to the ASM-bundle, matrix deposition and ASM hyperplasia.

Figure 1.3: Mechanisms involved in coupling ASM contraction with calcium excitation and sensitivity.



ASM contraction is dependent upon activation of actin and myosin cross-bridges and the phosphorylation of myosin light chain (MLC) by MLCK. This process is under the tight regulation of the calcium sensitivity and calcium excitation. Calcium sensitivity reflects the balance between MLCK and MLC phosphatase (P) and can be modulated by a number of mechanisms including RhoKinase and cAMP; the latter upregulated in response to activation of Gs-coupled receptors by agents such as -agonists. Calcium excitation i.e. the concentration of the free cytosolic calcium $[Ca^{2+}]_i$ is controlled by the influx/eflux of calcium from the intracellular store (sarcoplasmic reticulum) and extracellularly. In response to an agonist e.g. histamine via Gq-protein coupled receptors PIP_2 is converted by phospholipase (PL)C to IP3 and DAG. IP_3 activates the IP_3 receptor (IP_3R) on the sarcoplasmic reticulum and leads to release of intracellular stores. This in turn activates influx of extracellular calcium probably via store operated calcium channels (SOCC) (possibly via interactions between STIM-1 and Orai1), which can amplify the response. Following activation calcium homeostasis is restored by calcium pumps such as sarcoplasmic reticulum ATPase (SERCA). Importantly other potential mechanisms, some of which are shown, can modulate intracellular calcium concentration and modify the calcium sensitivity. Hence, it is important to note that this figure does not completely capture the complexity of the mechanisms involved.

1.4.2.2 A reflection of altered airway geometry/remodeling.

Airway remodeling is a central feature of asthma and refers to the changes in the structural components of the airway wall. The major features of airway remodeling include epithelial cell fragility, goblet cell hyperplasia, matrix deposition, myofibroblast hyperplasia, increased ASM mass and angiogenesis[76;284]. Studies of human disease have illustrated that some of the features of remodeling can occur early in disease in particular epithelial loss and basement membrane thickening[285]. Several studies have shown correlations with a number of the features of remodeling and AHR in univariate analysis[76], but whether these reflect causality or an epiphenomenon is unclear. Perhaps the most compelling is the association between ASM mass and AHR. Biopsy studies of asthma have consistently demonstrated an increase in the percentage of ASM mass compared to healthy subjects[32;116;286] due to a combination of ASM hyperplasia[88] and hypertrophy[76]. Mathematical modelling studies suggest that an increase in the content of ASM mass irrespective of hypertrophy or hyperplasia will increase the potential for airway narrowing[21]. In support of this view detailed bronchoscopic studies have attempted to define the relationship between airway dysfunction and features of remodeling; with ASM mass the feature most closely associated with airflow obstruction. However, to date no study has used a similar approach to unravel the association between AHR and remodeling.

The relationship between remodeling and AHR is complex. Indeed, paradoxically some features of remodeling may protect the airway from remodeling The beneficial effects include the potential for the airway to resist dynamic compression due to increased airway wall stiffness as a consequence of increased airway wall thickness, the theoretical attenuation of contraction by deposition of matrix around the ASM cells[265]thus providing an elastic impedance[268]and the potential increased elastic load on the muscle as a result of the distortion and folding of thickened remodeled tissue that occurs with ASM contraction[287]. Conversely, the structural changes of remodeling can have significant deleterious effects on airway resistance. Hyperplasia of the mucus-secreting goblet cells will contribute to luminal narrowing. An ASM layer of greater volume will narrow the airway lumen more than a lesser volume[20;288]. Furthermore,

mathematical models have predicted that an increase in the volume of the airway wall on the luminal side of the smooth muscle layer will greatly increase the effect of ASM contraction in narrowing the lumen of an airway[288-290]. Whereas, thickening of the outer adventitial wall may uncouple the airway from parenchymal elastic recoil forces and thus augment the ability of the wall to contract[291].

Imaging studies using computed tomography have been particularly informative of the global effects of airway wall thickening upon airway function. *Niimi et al* have demonstrated that the sensitivity (the point of initial rise in airway resistance during methacholine challenge) of airways in asthma is directly associated with eosinophilic airway inflammation; in contrast thickening of the airway wall in proximal airways was associated with reduced airway reactivity (the slope of the methacholine dose response airway resistance curve)[67]. Remodeling of the airway wall may therefore protect against the development of AHR. In contrast Beigelman-Aubry et al failed to demonstrate any difference in cross section airway calibre (airways >4mm^2 in size) on CT or lung attenuation before and after methacholine and after inhaled salbutamol in patients with mild intermittent asthma. However the degree of air trapping on expiratory CT scans did increase significantly after methacholine challenge suggesting that an important site of airflow resistance in asthma is in the small airways beyond the current resolving power of conventional CT[292]. A previous imaging study by Goldin et al also suggests that airways 2-5mm^2 in calibre are an important site of airflow limitation after methacholine challenge in asthma[293]

Taken together, the findings are consistent that the sum effect of airway remodeling changes in asthma is a greater degree of airway narrowing, which results in increased airway resistance. However, to date the literature on the relationship between AHR and remodeling is less clear. Importantly, remodeling is a dynamic process and one possible explanation for the apparent inconsistency in reports is the likelihood that in early stages of the disease components of airway remodeling exacerbate AHR but as the disease becomes progressive some of the features of remodeling may protect against

AHR. Therefore, longitudinal studies combining CT and parallel analysis of airway compartments in endobronchial biopsies before and after treatments that modulate airway inflammation and or airway wall remodeling are required to establish which components of the remodeling process are associated with AHR in asthma.

1.4.2.3 A consequence of airway inflammation.

A variety of allergen challenge studies have linked AHR with allergic airway inflammation and the late asthmatic response (LAR) and allergen challenge has been associated with eosinophilic airway inflammation in BAL and induced sputum[294-300]. However the extent to which the increased bronchial reactivity seen after allergen challenge and in animal models is related to the AHR seen in clinical disease is debatable. Despite intensive research over the last twenty years the extent to which AHR is caused by, or interacts with, airway inflammation and especially eosinophilic inflammation remains contentious. AHR and eosinophilic inflammation generally occur together. In the early bronchoscopy studies although there was a clear association between AHR and an airway eosinophilia there was little evidence of a correlation between the severity of the AHR and the number of BAL eosinophils [301]. Similarly in a study by Foresi et al of 15 asthmatics and 30 patients with seasonal allergic rhinitis a good correlation was seen between sputum eosinophils and AHR, but this was skewed by the inclusion of patients with seasonal rhinitis without asthma[302]. Some studies have seen a correlation. For example Jatakanon et al found a weak inverse correlation ($r= -0.4$) between the sputum eosinophil count and AHR in 35 stable asthmatics taking only β_2 agonists[303]. In our own experience of over 200 stable asthmatics attending our routine outpatient clinics (46% atopic and 44 % taking inhaled steroids) there was no relationship between log sputum eosinophil count and log PC_{20} in the whole group. However, there was a significant although weak inverse correlation in the atopic group ($r=-0.3$, $p<0.01$) in both patients taking inhaled steroids and those on β_2 agonists alone[220]. Therefore, a number of studies have demonstrated modest associations of AHR to methacholine with eosinophilic airway inflammation in sputum and broncho alveolar lavage fluid (BAL)[300;301] and indirect AHR has been shown to correlate more closely with airway inflammation[304;305].

Current evidence supports the idea that AHR and eosinophilic airway inflammation are independently regulated but closely interrelated, a view supported by a factor analysis undertaken by Rosi et al in 99 mild asthmatics[306]. This would predict that in a cross-section of patients, for a given degree of inflammation, marked differences in AHR could result. This is consistent with the observation that eosinophilic inflammation can occur without AHR as in non-asthmatic eosinophilic bronchitis and marked AHR can occur in the context of minimal airway eosinophilia, as seen in non-eosinophilic asthma. However, inhaled corticosteroids[307] and allergen avoidance[308] have been shown to improve both AHR and airway inflammation, which suggests that within an individual, changes in AHR may mirror changes in eosinophilic airway inflammation to the extent that airway inflammation could be used longitudinally to guide asthma management.

1.4.2.4 Non-asthmatic eosinophilic bronchitis: A powerful disease control model

Non-asthmatic eosinophilic bronchitis (EB) is a condition of unknown aetiology in which patients present with a chronic, minimally productive cough and are found to have an airway eosinophilia (>3% in sputum), but without wheeze, shortness of breath, variable airflow obstruction or AHR[309]. It has been found in 13% of patients who present with cough[310]. The natural history of the disease is unclear, although in some cases persistent airflow obstruction may occur[311;312] While EB is interesting in its own right, it has particular significance in offering clues as to why eosinophilic airway inflammation in some, but not all, individuals leads to asthma[313].

We have investigated the immunopathology of EB in comparison with that of asthma, and have confirmed that EB is characterised by a submucosal eosinophilic inflammation and sub-epithelial thickening of the collagen layer[105;314]. The expression of T-cell activation and of chemokine receptors and T-cell cytokines was also similar to those in asthma, with both conditions showing a Th_2 pattern of T-cell activation[315]. Indeed, the only immunopathological difference we observed between the two conditions was infiltration of the airway smooth muscle (ASM) by mast cells in asthmatics with increased expression of IL-13, but not in patients with EB or in normal subjects[105;316]. There was also a significant correlation between the number of mast cells in the ASM

and AHR[105]. This was despite the observation that the overall numbers of mast cells in the airway lamina propria in the three groups was the same, and our previous demonstration that EB is characterised by increased amounts of histamine and prostaglandin D_2 in the sputum, suggesting the presence of activated mast cells in the epithelium[317]. Strikingly, there was no infiltration of the ASM by eosinophils or T cells in asthma or EB. The immunopathological differences between asthma and EB are summarized in Table 1.1.

Importantly, in addition to mast cell infiltration into the ASM-bundle a recent CT study has shown that the proximal airway wall is not thickened in EB [318] compared to patients with asthma. However this study was limited by the fact that the duration of disease was only 8 months (median value) in patients with EB[319].

Therefore mast cell infiltration in the ASM in asthma may be an important mediator of AHR in contrast the association of proximal airway geometry upon AHR in asthma is less clear.

Table 1.1: Comparative Immunopathology of Asthma and Non Asthmatic Eosinophilic Bronchitis.

	Asthma	**Non asthmatic Eosinophilic bronchitis**
Airway Hyper responsiveness	**Present**	**Absent**
Eosinophilic airway Inflammation in sputum, BAL and bronchial biopsies	**Present**	**Present**
Mast cells in the ASM	**Increased** compared to control	**Not** Increased compared to control
Il-13 expression in the airway subepithelium and peripheral blood	**Increased** compared to control	**Not** Increased compared to control
Vascular remodeling	**Increased** compared to control	**Unknown**
Reticular basement membrane thickening	**Increased** compared to control	**Increased** compared to control
Increased ASM mass/matrix deposition and glands	**Increased** compared to control	**Unknown**
Reduced proximal airway luminal patency on CT.	**Present** compared to control	**Unknown**
Evidence of small airways disease on HRCT	**Present**	**Present**

1.4.2.5 Mast cells in the airway smooth muscle: Fundamental to the pathogenesis of AHR in asthma

Despite the premise that *in vitro* ASM sensitivity may account for *in vivo* responsiveness to bronchial challenge agents in man, previous studies comparing *in vivo* AHR and *in vitro* responsiveness to methacholine suggest that factors other than intrinsic ASM sensitivity regulate AHR[320].

In asthma the ASM-bundle is infiltrated by mast cells, predominately of the chymase-positive phenotype[77;105;110;111;113;321;322]. Mast cells adhere avidly to ASM in part via a tumour suppressor in lung cancer-1 (TSLC-1) heterophilic adhesion molecule and in part via an unidentified Ca^{2+} independent pathway[323]. Mast cell number correlated inversely with AHR[105] and positively with the bronchoconstrictor response to a deep inspiration[324] suggesting that mast cell-ASM cell interactions are likely to be central in the development of the disordered physiology in asthma. The latter finding is augmented by a previous study in diabetic autonomic neuropathy that suggested that airway responses to deep inspiration are independent of vagal tone[325]. One post mortem study of fatal and non-fatal asthma has shown that there was a marked increase in mast cell degranulation in the ASM-bundle in both the large and small airways[43]and another demonstrated that increased numbers of mast cells (degranulated and intact) are associated with increased ASM shortening in fatal asthma[112].

Activation of the inflammatory cells within the ASM-bundle would be predicted to have important consequences on ASM function. Following mast cell degranulation the mediators histamine, PGD_2 and LTC_4 are released which are all potent agonists for ASM contraction[326]. Mast cell cytokines may further contribute to AHR. The mast cells in the ASM-bundles in asthma, but not in COPD, express IL-13[327]. IL-13 has been shown to attenuate relaxation to β-agonists and augment contractility to acetylcholine[328-330]. Similarly mast cell proteases may be important in modulating ASM contractility and AHR[331;332].

The interactions between inflammatory cells and ASM cells may have more long-term consequences. Mast cells co-cultured with ASM promote ASM differentiation with

increased α-smooth muscle actin expression and we have recently demonstrated that mast cells within or in close proximity to the ASM bundle increased the % of high intensity actin expression *in vivo* (figure 1.4)[333]. Similarly, mast cell differentiation towards the chymase-positive phenotype observed within the ASM-bundle may be mediated by mast cell-ASM interactions. Increased ASM mass is a well-established feature of asthma[32]. A number of mast cells mediators including histamine[334], tryptase[335]and LTD_4[156] promote ASM proliferation. Alternatively increased ASM mass may be a consequence of recruitment of ASM progenitors. This view is supported by the increased number of fibrocytes that migrate into the airway following allergen challenge[180]. Recent evidence suggests that ASM migration towards the ASM-bundle is mediated by activation of CCR7 by ASM and mast cell-derived CCL19[185].

Therefore mast cell interactions with ASM in asthma are likely to be important in the development of AHR and may play a critical role in the development of increased ASM mass and the development of fixed airflow obstruction seen in severe disease. Mast cell airway smooth muscle interactions are summarised in (figure 1.5).

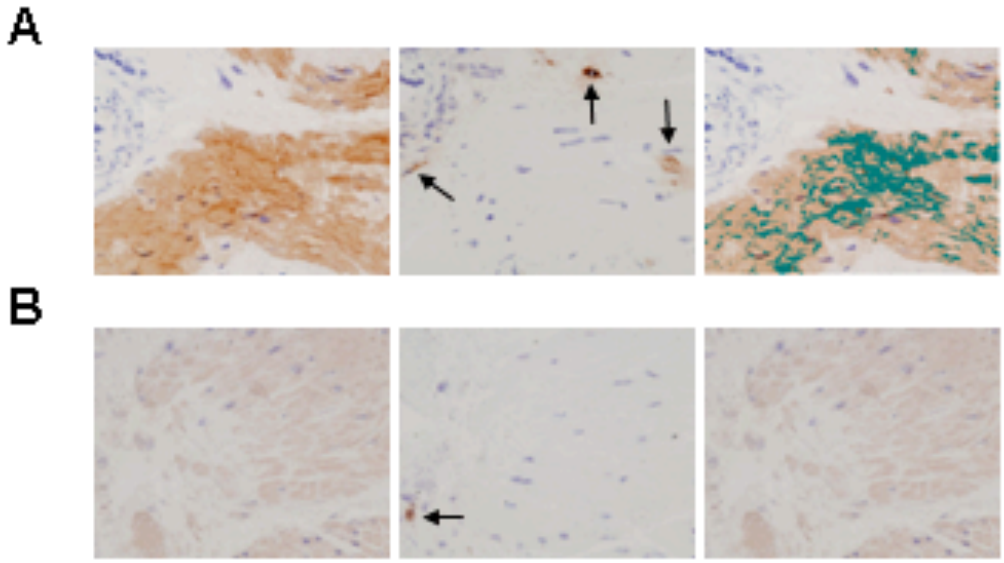
The infiltration of mast cells into tubular visceral structures has been associated with organ dysfunction in variety of other disease systems. Mast cells in close proximity to colonic nerves have been implicated in the pathogenesis of irritable bowel syndrome[336] and mast cells within the myometrium have been associated with pre eclampsia[337]. Indeed we have recently demonstrated that AHR was increased independent of atopy in women who had previously had pre eclampsia in pregnancy compared to women that had not had pre eclampsia[338]

Summary (1.4.1-1.4.2.5)

AHR has a clinical utility in combination with other measures in the diagnosis of asthma. Recent studies have given us a real opportunity to unravel the relationship between AHR, airway inflammation and remodeling and have given prominence to the role of mast cell-ASM interactions. However, the challenge for clinicians is to be able to understand the mechanisms driving AHR in an individual asthmatic and to select specific therapy appropriate to normalise airway function.

Figure 1.4: Increased ASM α-SMA expression *in vivo* localized with mast cells

a) *Photomicrographs of a bronchial biopsy from an asthmatic illustrating ASM-bundles stained for α-SMA (left), sequential tryptase stained section revealing 1 mast cell within the ASM-bundle and 2 in the 30 m perimeter of the bundle (middle), and high intensity thresholded α-SMA stained bundle covering 8.76% of the total ASM-bundle area (right) (x400).* ***b)*** *Photomicrograph of another asthmatic again illustrating α-SMA stained ASM-bundle (left), sequential tryptase stained section revealing 1 mast cell in the 30 m perimeter and none within the ASM-bundle (middle), and high intensity thresholded α-SMA stained ASM-bundle covering 0% of the total ASM-bundle area (right) (x400).* ***c)*** *Correlation between the percentage of high intensity α-SMA staining and the number of mast cells in the ASM-bundle alone (r=0.87; p=0.0003) and* ***d)*** *together with the number of mast cells in the 30 m perimeter of the ASM-bundle (r=0.87; p=0.0002) n=12 asthmatics*



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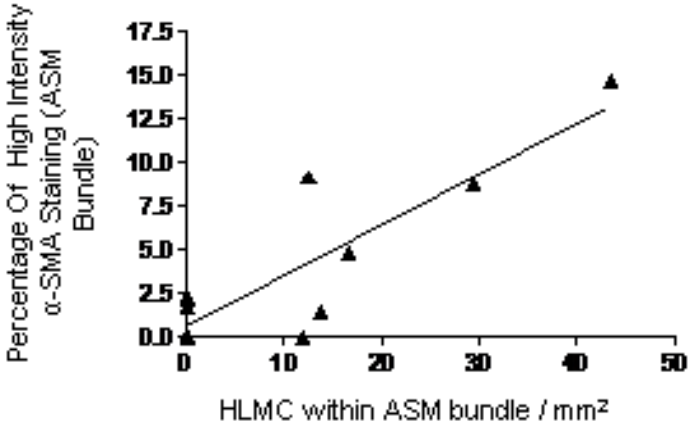

Percentage Of High Intensity α-SMA Staining (ASM Bundle)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
10
20
30
40
50
HLMC within ASM bundle / mm²

D

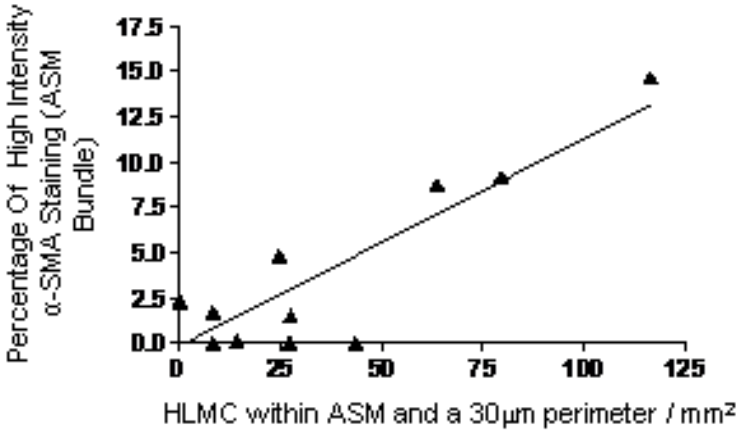
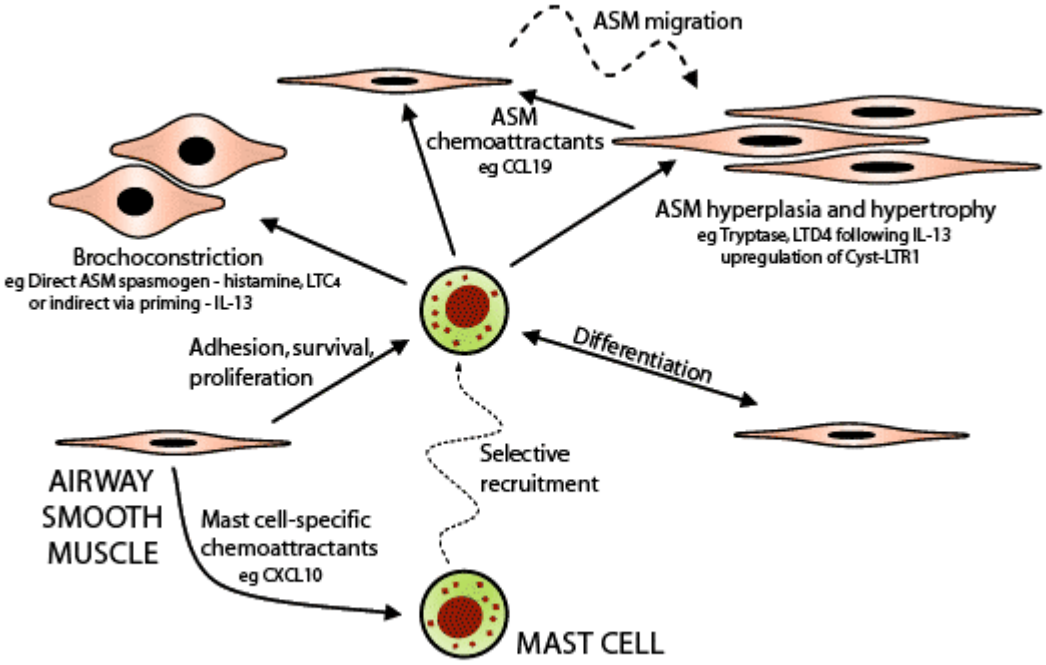

Percentage Of High Intensity α-SMA Staining (ASM Bundle)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
25
50
75
100
125
HLMC within ASM and a 30µm perimeter / mm²

Figure 1.5: Mast cell-ASM interactions



Mast cells are recruited to the ASM under the influence of ASM-derived chemoattractants and adhere to ASM. In the ASM-bundle there is an appropriate environment to support mast cell survival and the cells interact resulting in cellular differentiation, ASM hyperplasia, recruitment of ASM progenitors and ASM contraction either directly or indirectly.

1.5 Airflow limitation in asthma

1.5.1 Determinants of airflow limitation in asthma.

Cross sectional studies of asthma have consistently demonstrated that lung function is less than predicted[339-341]. Airflow limitation is a well recognised feature of asthma and has been described in a number of large epidemiological studies independent of disease severity; however it is commoner in patients with persistent symptoms and severe asthma. A subgroup of asthmatics also develop fixed airflow limitation characteristic of COPD[17;18;45;48]. Furthermore the rate of decline in FEV_1 is increased in asthmatics, independent of cigarette smoking[48;49;341;342] and seems to be greater in patients with newly diagnosed asthma[341]. A variety of factors have been independently linked to airflow limitation and decline in FEV_1 in asthma including smoking, age of onset, duration of disease, symptom severity, atopy, AHR and baseline level of lung function at school age (reviewed in[343]). Exacerbations particularly severe exacerbations seem to be important in promoting accelerated FEV_1 decline in asthma[344]. Interestingly many of these risk factors are repeatable in severe asthma however ethnicity and socioeconomic status may also be important factors in severe disease[345]. More recently with the advent of induced sputum as an indirect measure of airway inflammation; sputum neutrophilia[346;347] has emerged as an important cofactor in the development of airflow limitation in asthma. Similarly Ten Brinke et al demonstrated that sputum eosinophilia predicted fixed airflow obstruction in asthma using a multivariate analysis[348] and that seropositivity to *C Pneumoniae* may promote fixed airflow obstruction in non atopic asthma[349].

1.5.2 Evidence to support a role for remodeling and airflow limitation

A number of cross sectional studies of airway remodeling have found a relationship between different aspects of cellular and structural remodeling with airflow limitation in asthma. Pretolani et al demonstrated that fibroblast numbers in the subepithelium and ASM size best modelled post bronchodilator FEV_1 in a multivariate analysis[76]. One limitation of this study was that the cohort of patients with refractory asthma had severe airflow limitation, which may have indicated that treatment had not been optimised.

More recently Sterk et al have demonstrated that cellular inflammation within the airway wall due to CD8+ T cell infiltration is associated with FEV_1 decline in asthma[350].

In keeping with these observations a number of imaging studies of proximal airways have demonstrated that proximal airway wall thickening is associated with airflow limitation in asthma[63;68;96]. Kasahara et al demonstrated that RBM thickening correlated with wall thickness and percentage wall area on HRCT imaging and with airflow limitation in asthma[351]; further studies linking structural remodeling with global airway wall thickening are required.

Few studies have examined the impact of remodeling of small airways upon airflow limitation. Hamid et al have demonstrated that increased ASM mass in small airways obtained via transbronchial biopsy is associated with FEF_{25-75} in asthma in contrast to matrix deposition[114]. Ueda et al have demonstrated that air trapping and low attenuation areas on expiratory and inspiratory HRCT scans is associated with airflow limitation in asthma[70]. However low attenuation areas on CT in asthma have not been validated with corresponding pathology in contrast to COPD[71].

Summary (1.5.1-1.5.2)

Airflow limitation is a well recognised feature of asthma and is associated with a variety of factors including age of onset, duration of disease, airway inflammation, persistent symptoms, AHR and exacerbations. Structural remodeling of the airway wall and global airway wall thickening on HRCT have been associated with airflow limitation in asthma.

1.5 Hypothesis

1) I hypothesise that structural remodeling of the airway wall of proximal conducting airways in asthma (airway wall thickening, increased ASM mass, vascular remodeling, glandular hyperplasia, reticular basement membrane thickening and matrix deposition in the lamina propria) is dissociated from AHR and associated with airflow limitation.

2) I hypothesise that one of the key determinant of AHR in asthma is cellular infiltration of the airway smooth muscle with mast cells.

3) I hypothesise that the patency of the airway lumen in proximal airways is a key factor regulating AHR in asthma.

4) I hypothesise that fibrocytes are increased in the lamina propria and smooth muscle bundle in asthma and may contribute to increased ASM mass.

5) I hypothesise that proximal airway wall thickening is dissociated from eosinophilic airway inflammation in severe asthma; assessed using induced sputum.

1.6 Aims

1) ***To demonstrate that airway wall structural remodeling is dissociated from AHR in asthma and associated with airflow limitation.***

   To compare and contrast features of structural remodeling (vessels, airway smooth muscle, glands, matrix, reticular basement membrane thickening) and cellular remodeling (granulocytic inflammation of the airway wall, cellular infiltration of the ASM, fibrocyte numbers in the ASM and lamina propria) using endobronchial biopsies in asthma, non asthmatic eosinophilic bronchitis and healthy controls. To assess the relationship between remodeling, AHR and airflow limitation in asthma.

2) ***Define the importance of altered luminal patency due to airway wall thickening and in asthma.***

   To validate the quantitative measurement of airway geometry on computed tomography images and determine the repeatability and limit of resolution.
   To quantitatively assess the static geometry of the proximal airway wall and luminal patency in asthma and non asthmatic eosinophilic bronchitis using multislice computed tomography. To define the association of static geometry with AHR and airflow limitation in asthma.
   To quantitatively assess the static geometry of the proximal airway wall and luminal patency using mutislice computed tomography, in severe asthmatics in the presence or absence of eosinophilic airway inflammation.

2 Methods

2.1 Clinical

2.1.1 Peripheral blood tests and skin tests

Venous blood samples were taken to measure peripheral blood eosinophil count, total IgE. Atopy was assessed by skin prick tests to *Dermatophagoides pteronyssinus*, cat fur, dog, grass pollen and *Aspergillus fumigatus* solutions with normal saline and histamine controls (Bencard, UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal >2mm more than the negative control.

2.1.2 Exhaled Breath Nitric Oxide

Exhaled nitric oxide concentration was measured at 50 $mL \cdot s^{-1}$ using an online chemiluminescence analyser (NIOX; Aerocrine, Stockholm, Sweden) prior to any other measurements

2.1.3 Spirometry

Spirometry was performed with a Compact Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Bronchodilator reversibility was assessed 15 minutes after administration of 2.5mg salbutamol nebulised via a Flaem Nuova Type II nebuliser (Deva Medical, Runcorn, Cheshire) with a median particle size of 2 μm and the patient breathing tidally or 200μg salbutamol inhaled via a volumatic. FEV_1 was recorded as the better of two successive readings within 100 mL.

2.1.4 Airway responsiveness

Using the standard tidal breathing method the concentration of methacholine causing a 20% fall in FEV_1 was recorded as the $PC_{20}FEV_1$[241]. In brief, following the measurement of the baseline FEV_1 subjects inhaled saline followed by doubling concentrations of methacholine 0.03-16 mg/ml via a Wright's nebuliser (flow 0.13ml/min driven by dry compressed air) (gift from Fisons, Leicestershire, UK). Additional concentrations of methacholine were used in some studies of 32, 64, 128 mg/ml. The subject was instructed to breathe quietly (tidal breathing) for 2 min with a noseclip. The FEV_1 is measured 30 and 90 s after the nebulisation is completed. If the FEV_1 fell less than 20% the procedure was repeated with the next highest concentration. If the FEV_1 fell more than 20% from baseline (or the highest concentration has been given), no further methacholine was given. Methacholine (histamine) $PC_{20}FEV_1$ concentration was calculated by linear interpolation of the log dose response curve.

2.1.5 Sputum induction

Instructions for Patients

Prior to commencing the induction the procedure was fully explained to the patient including:

Instruction on spitting out saliva generated during inhalation of saline into a "discard" vessel.

Instruction about blowing their nose and rinsing their mouth and swallowing the water prior to trying to expectorate sputum. (It is important that the subject moves quickly through this procedure to prevent loss of sputum due to swallowing).

Instruction on how to expectorate effectively. It is necessary to explain and demonstrate the technique for coughing up sputum and moving sputum from the back of the throat, forward to the specimen container.

A reminder not to swallow the sputum as it comes up the bronchial tree.

Guidance on posture: sitting straight upright during nebulisation, and leaning forward during expectoration.

Protocol

Subjects were pre-treated with either inhaled salbutamol 200 μg or nebulised salbutamol 2.5mg 10-30 minutes before sputum induction to minimise bronchoconstriction. Sputum was induced using 3, 4 and 5% saline inhaled in sequence for five minutes via an ultrasonic nebuliser (Medix, Harlow, UK; output 0.9 ml/min; mass median diameter 5.5 μm). Tidal breathing was employed, taking a slightly deeper breath every minute. After each inhalation patients blew their noses and rinsed their mouths to minimise nasal contamination and expectorated sputum into a sterile pot. FEV_1 was measured after each inhalation. If the FEV_1 fell by more than 10% but less than 20%, the same concentration of saline is administered. If the FEV_1 fell by more than 20% of the best post-bronchodilator value, or if significant symptoms occurred, the nebulisation was stopped and the patients were treated with repeat short-acting β-agonist. (Figure 2.1)

Safety procedures during the induction.

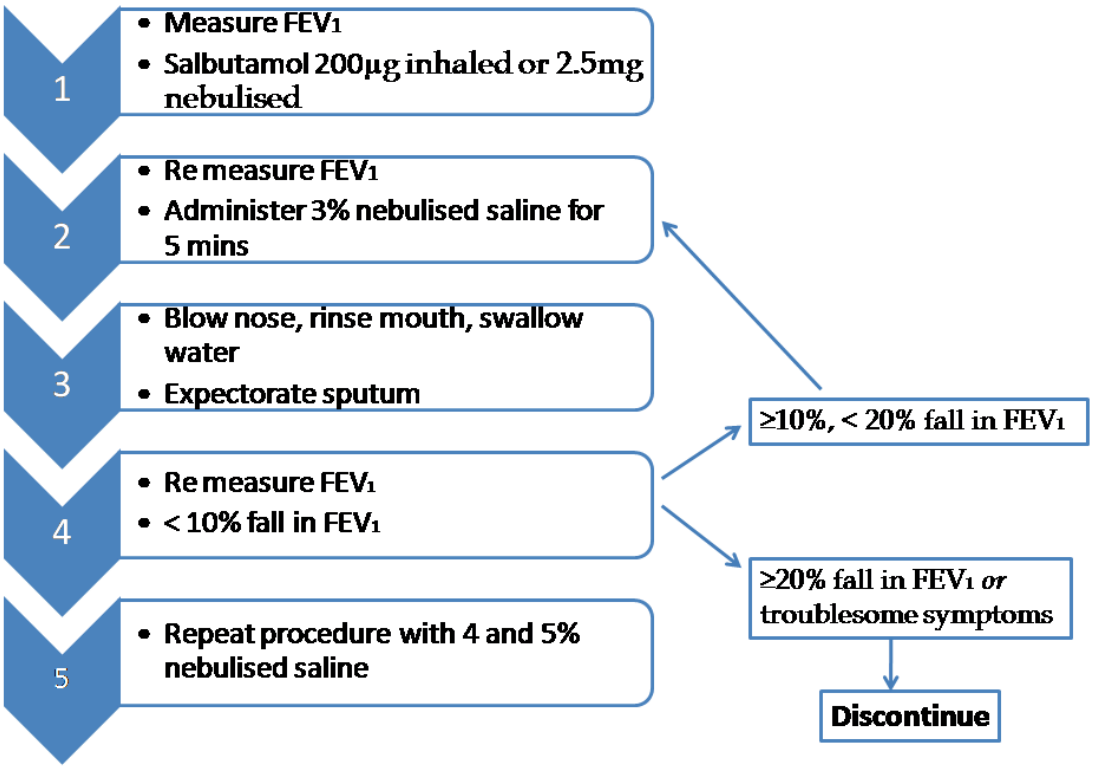
Inhaled hypertonic saline is a bronchoconstrictor stimulus so sputum induction using ultrasonically nebulised hypertonic saline should be carried out with care. The usual laboratory resuscitation apparatus plus nebulised salbutamol was readily available. A doctor either performed the procedure or was nearby during each procedure.

2.1.6 Bronchoscopy

Bronchoscopy was performed as a day case procedure in line with the British Thoracic Society guidelines[352]. After obtaining written informed consent subjects were premedicated with nebulised 2.5mg salbutamol. Topical lignocaine gel is applied inside the nose. Sedation was offered to the subjects and was given in boluses of 2.5mg midazolam to a maximum of 5mg. Supplemental oxygen was supplied and pulse oximetry monitored throughout the procedure. 4ml of 4% lignocaine was administered

via the bronchoscope to the vocal cords or via the transtracheal route and 3 aliquots of 2ml of 2% was applied to the trachea, right and left main bronchi. Four-six bronchial biopsies were taken from the segmental and subsegmental carina from the right lung using a 20-cupped biopsy forceps.

Figure 2.1: Sputum Induction Protocol



2.1.7 Computed Tomography

All patients were instructed to take a repeatable breath hold to total lung capacity prior to the scan due to the known association of lung volume and cross sectional geometry of airways. CT scans were not gated to the respiratory cycle and were acquired immediately after the patient had been asked to take a deep breath in and hold their breath in the supine non prone position.

Long acting bronchodilators were taken by patients who were normally on them as maintenance asthma therapy on the morning of their CT scan typically 2-4 hours prior to their scan. Therefore the effects of increased airway smooth muscle tone were minimised.

2.1.7.1 Limited Computed Tomography of the RB1 (apical segmental bronchus, right upper lobe) Bronchus

We devised a limited CT imaging protocol to capture the apical segmental bronchus of the right upper lobe (RB1) bronchus. We chose this airway as a marker of global airway wall remodeling because i) reference measures of geometry for the airway have been published in asthma and COPD[65;68]; ii) To standardise geometry measurements to a single anatomical location in a proximal conducting airway; with a similar calibre to those sampled using endobronchial biopsy specimen; iii) RB1 is perpendicular to the CT scanning plane in the supine position and is therefore sectioned non obliquely; thus minimising the bias of oblique orientation upon airway geometric measures and iv) RB1 geometry has been shown to correlate with airway wall geometry of other proximal airways in COPD[65] and gas trapping on expiratory CT scan in asthma[96].

For archived high resolution CT scans (HRCT) scans from the difficult asthma clinic we analysed scans that had captured the apical segmental (RB1) bronchus.

2.1.7.2 RB1 imaging protocol

Prior to CT imaging a scout CT radiograph of the thorax was taken in the supine position at full inspiration. A standard scanning block of 5.3cm was superimposed on to the scout film with the superior edge at the level of the aortic arch and inferior edge 1

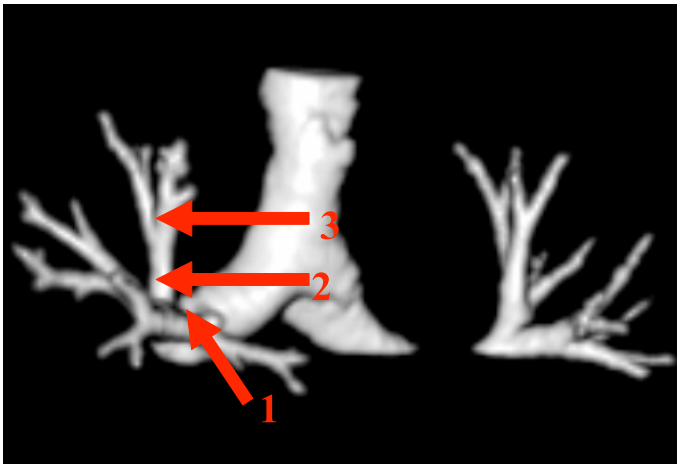
cm below the main carina. The same block was used to define the scanning range in all patients and in patients undergoing multiple limited CT scans.
Subjects then had a limited CT scan of the RB1 (apical segmental bronchus; right upper lobe) bronchus scanning from the aortic arch to the carina [5.3 cm scan length]. CT scanning was performed with a Siemens Sensation 16 CT scanner. Scans were obtained at 16x0.75 collimation, 120 kVp, 50 mAs with a table speed of 13.5 per 0.5 second scanner rotation, and scan time of 2.81 seconds. Images were reconstructed at 0.75 mm spacings using 295 mm field of view with a 512 x 512 matrix and a very sharp reconstruction algorithm (B70-f). RB1 tomographic anatomy is shown in figure 2.2.

2.1.7.3 Standard Thoracic HRCT

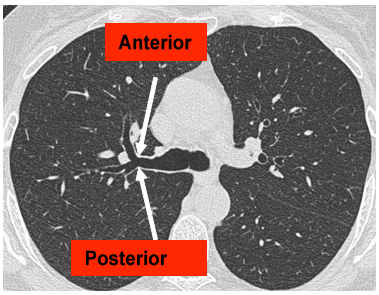
HRCT scanning was performed using a Siemens Sensation 16 slice scanner at 120kVp, 140mAs, 16x1mm collimation with 10mm slice intervals.

All CT scans had been reported by an experienced radiologist prior to analysis.

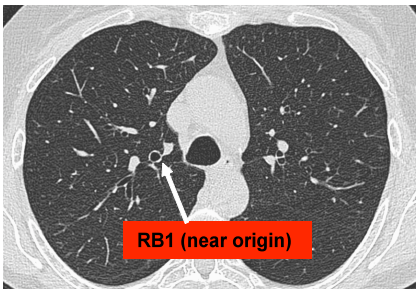
Figure 2.2: RB1 bronchus tomographic anatomy: Limited CT Scan



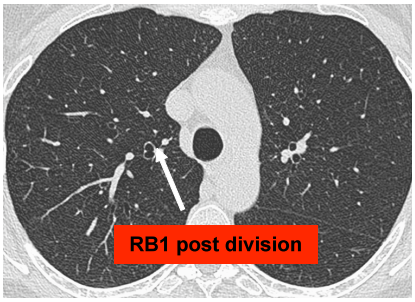
1)



2)



3)



a) 3D segmentation of a limited CT scan of the RB1 bronchus. CT slices at 1) prior to the origin of RB1 at the level of the anterior and posterior divisions of the right upper lobe; 2) The origin of the RB1 bronchus and 3) Immediately post division of RB1 into a 4^{th} generation airway.

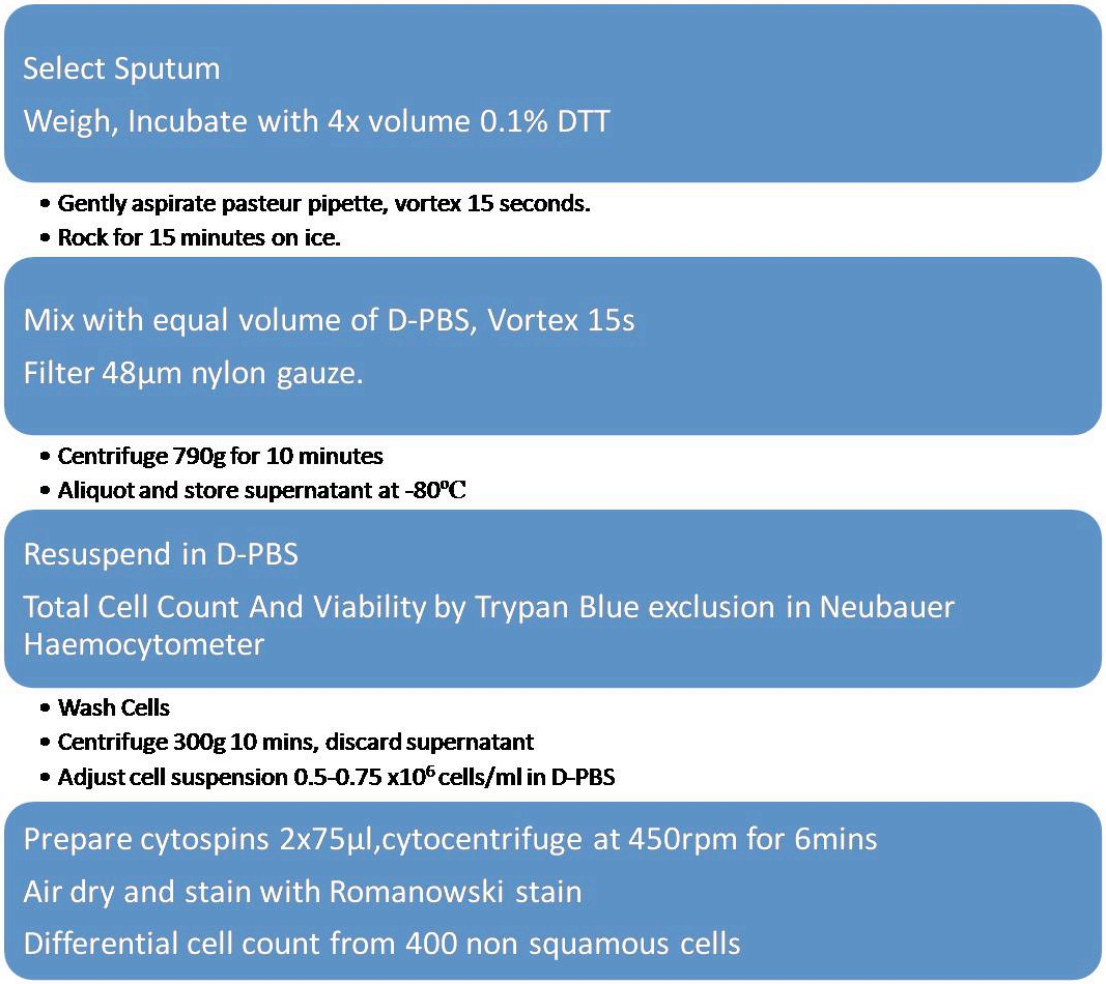
2.2 Laboratory

2.2.1 Sputum processing

Protocol sputum processing

Sputum free from salivary contamination was selected and weighed. To the selected sputum was added 4x volume/weight of 0.1% dithiothrietol (DTT) (Sigma, Poole Dorset). The sputum was dispersed by gentle aspiration into a Pasteur pipette, vortex for 15 s and 15 mins rocking on a bench spiromix. After the addition of an equal volume of Dulbecco's phosphate buffered saline (D-PBS) (Sigma, Poole, Dorset) the sputum suspension was filtered through 48μm nylon gauze and centrifuged 2000rpm (790g) for 10 mins. The sputum supernatants was removed and stored at -80^{o}C for future mediator assay. The cell pellet was resuspended in a small volume of PBS. An aliquot was removed and a total cell count, squamous cell contamination and viability were assessed using a neubauer haemocytometer by the trypan blue exclusion method. The cell suspension was adjusted with PBS to 0.5-0.75 x10^{6} cells/ml and cytospins were prepared from 75μl aliquots at 450rpm (18.1g) for 6 mins using a Shandon III cytocentrifuge) (Shandon, UK). The cytospins were stained in neat Romanowski stain for 5 mins and fixed in dilute stain for 25 mins. A differential cell count was obtained by counting >400 non-squamous cells on a Romanowski stained cytospin (figure 2.3).
Romanowski stain preparation:
Dissolve 1.5g Azure-B-thiocyanate in DMSO at 37^{o}C and 0.5g Eosin in 300ml methanol at room temperature. Slowly add the Azure blue solution to the Eosin and store away from light.
Dilute Romanowski stain:
62 ml 10mM HEPES buffer pH 7.2
3.5 ml DMSO
4.6 ml Romanowski stain

Figure 2.3: Sputum Processing Protocol



2.2.2 Biopsies

2.2.2.1 Fixation, processing and embedding in glycomethacrylate and paraffin

Protocol glycomethacrylate blocks

Mucosal biopsies were immediately transferred into ice-cooled acetone containing the protease inhibitors (20mM) (PMSF) (2mM) for fixation, stored at -20 C for 24h. The fixative was replaced with acetone followed by methyl benzoate at room temperature for 15 min each. The biopsies were infiltrated with 5% methyl benzoate in glycol methacrylate (GMA solution A, Polysciences, Northampton, UK) at 4 C, 3x2 hours and then embedded in a solution of GMA A 10mls: GMA solution B 250 ls: Benzoyl peroxide 45mg (which acts the catalyst for polymerisation). The blocks were polymerised at 4 C overnight and kept in dry airtight boxes at -20 C.

Immunohistochemistry

Buffers and solutions

Tris Buffered Saline pH 7.6

Sodium chloride 80g

Tris 6.05g

1M hydrochloric acid 38mls

Distilled water 10L

Mix buffer salts and acid in 1L of distilled water, adjust pH to 7.65 and add to remaining 9L of water to give a final pH of 7.6

Blocking medium

Dulbecco's modified Eagles medium 80mls

Fetal calf serum 20mls

Bovine serum albumin 1g

Tris HCl Buffer pH 7.6

0.2M Tris 12mls

0.1M hydrochloric acid 19mls

Distilled water 19mls

Mix all reagents together, adjust pH to 7

Protocol

Two-micrometer sections were cut, floated on 0.2% ammonia solution in water for 1 min and dried at room temperature for 1-4h. The technique of immunostaining applied to GMA embedded tissue has been described previously[353]. Briefly, slides were pre-treated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide to inhibit endogenous peroxide. After 2x 5min washes in TBS pH 7.6, blocking medium consisting of Dulbecco's MEM, 10% FCS and 1% BSA was applied for 30 min. Sections were then incubated with the primary antibody for 16-20h overnight at room temperature at appropriate concentrations (Table 2.1). Bound antibodies were labelled with biotinylated rabbit anti-mouse Fab fragments (Dako Ltd., Ely, Cambridgeshire, UK) during a 2h incubation, and demonstrated using the streptavidin-biotin-peroxidase detection system (Dako Ltd). Aminoethylcarbazole (AEC) was applied as the chromogen, which gives a red reaction product. Sections were counterstained with Mayers haematoxylin. Appropriate control sections were similarly treated either with the primary mAb omitted or in the presence of an unrelated antibody of the same isotype (IgG_1 Sigma, Ltd).

Table 2.1: Antibody source and concentration used for immunohistochemistry

Antibody	Clone	Epitope Stained	Source	Dilution
MBP	BMK-13	Major Basic Protein	CALTAG	1:25
Tryptase	AA1	Mast cell tryptase	DAKO	1:1000
Neutrophil Elastase	NP57	Neutrophils	DAKO	1:1000
Collagen 3	FH-7A	Collagen Type 3	SIGMA	1:8000
Collagen 1	5D8-G9	Collagen Type 1	CHEMICON	15:1000
CD34	B1-3CS	CD34	ABCAM	1:30
α-SMA	1A4	Smooth muscle actin	DAKO	1:100
EN4	EN4	CD31/PECAM-1	MONOSAN	1:20
IgG1		Isotope control Ig	SIGMA	1:60
IgG2a		Isotype control Ig	DAKO	1:20
Biot-rab antiMo		Second stage antibody	DAKO	1:300
StABC-HRP		Third stage antibody	DAKO	1+1:200

2.2.3 Quantification of airway structure and cellular localisation.

2.2.3.1 Image analysis and quantitative morphometry

Morphometry was assessed by computer-assisted image analysis on hematoxylin-stained sections and α-smooth muscle actin stained sections for airway smooth muscle analysis. Total biopsy area was determined in at least two non-contiguous tissue sections from the same biopsy, and values are expressed in mm^2. The area of the lamina propria was derived by subtracting the ASM area, glandular area, epithelial area and area of vessels and lymphatics from the total biopsy area

2.2.3.2 Measurement of ASM area

We used the Cavalieri method to estimate the volume of ASM in 8 patients with EB[354]. In brief a single biopsy was cut into 28 sections 2μm apart giving a reference volume of 56μm and sections were counter stained with Mayer's haematoxylin. The reference volume was then interrogated at 5 systematically uniform random sections (SURS) by generating a random number between 1-11.2 and subsequently adding 11.2 iteratively 4 times to the original random number. A point counting system was used to determine the total area of the section and the ASM area for the 5 SURS. The top right quadrant of each cross was used as the zero dimensional reference point. Point counts were converted into section areas by multiplying the total number of points counted Σp, by the area per test point a(p).

The Cavalieri volume was then estimated by multiplying the distance between sections **t** by their total cross sectional area:

$$\mathbf{Vref = t^* a(p)^* \Sigma p}$$

Using this method the mean (sem) volume fraction of ASM was 0.23(0.05) $mm3/mm^3$.

We found an excellent correlation between this volume fraction and the mean area of ASM measured using image analysis planimetry in the individual SURS
rs=0.85; p=0.01.

We also found an excellent correlation between ASM area estimated using α-smooth muscle actin staining and haematoxylin staining in the SURS. $r=0.97$; $p<0.0001$.

We therefore assumed that α-smooth muscle actin staining of ASM was a reasonable estimate of ASM mass for the purpose of this study and used the ASM area in α-SMA stained sections as an estimate of ASM mass. This is in line with previous reports[76;355].

2.2.3.3 Measurement of collagen 3 deposition.

Intensity of matrix deposition in the lamina propria of collagen 3 was assessed qualitatively using a semi quantitative score (0-3) as previously described and in the ASM as either present(1) or absent(0) by two blinded investigators (VM,SS)[76]. The mean of the two investigators scores was taken as the final score.

For quantitative assessment of matrix deposition in the subepithelium a thresholding technique was developed based upon the hue, saturation and intensity (HSI) of collagen 3 staining. The HSI Colour system was defined by a scale of 0-255 for hue, saturation and intensity. Initially the background noise was defined by assessing the hue, saturation and intensity of collagen 3 deposition in the lamina propria in 4 patients with a lamina propria semi quantitative score of 3, 2 & 1 respectively. Sections were acquired at x25 magnification, white balance corrected and pixels of representative matrix staining selected until high intensity matrix staining in the lamina propria was appropriately thresholded. Areas of matrix staining in glands and the ASM were manually removed to give the area of matrix in the lamina propria. A minimum of two non contiguous sections were thresholded for each patient. The median (IQR) of lower and upper limit of HSI were then defined from the 12 validation patients. The lower quartile of hue [upper limit 10; lower limit 0], maximum saturation [upper limit 255; lower limit 33] and median intensity [upper limit 169; lower limit 94] were then selected as the final threshold to give a threshold that appropriately captured highly saturated red light. The final threshold was then applied to the isotype control slides of each of the 12 validation patients and the % area of matrix in the lamina propria in the isotypes defined. We then calculated the mean % isotype staining and the set the limit

of noise with the final threshold as the mean+2SD of the final %. The mean+2SD image noise was 0.88%. (Figure 2.4)

All biopsies were subsequently thresholded using the final threshold and the noise % was subtracted from each biopsy. The mean % area of collagen 3 in the lamina propria on 2 sections at least 20μm apart was taken as the final % area of collagen 3 in the lamina propria.

We tested the final threshold by comparing the semi quantitative matrix score with the thresholded % area of matrix in the lamina propria. We found that there was an excellent correlation ($r_s=0.83$; $p<0.0001$) and that the regression line crossed the x-axis at a SQS value of 1.01 suggesting that our final validated threshold identified matrix staining corresponding to a SQS of </= 1 as background noise (Figure 2.5).

The repeatability of the semi quantitative and quantitative matrix analysis is shown in table 2.2.

Figure 2.4: Thresholding of collagen 3 in the lamina propria

a) Collagen 3 stained section at x25 magnification. b) Threshold applied to collagen 3 (red colour) in the lamina propria. In this example the % of collagen 3 in the lamina propria was 15.3% after correcting for isotype noise and the semi quantitative collagen 3 score for matrix intensity measured by 2 observers (VM&SS) was 3.

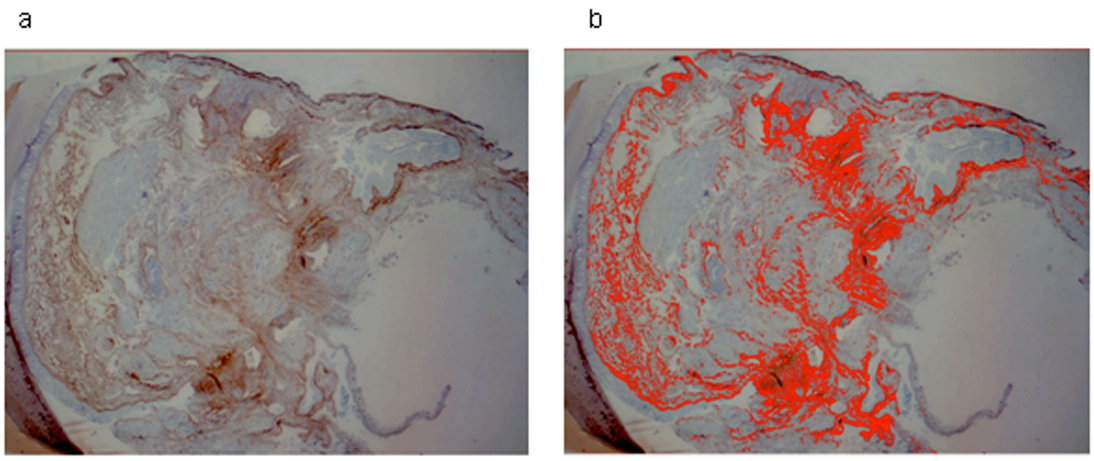


Figure 2.5: Correlation between semi quantitative collagen 3 score in the lamina propria and thresholded collagen 3 %

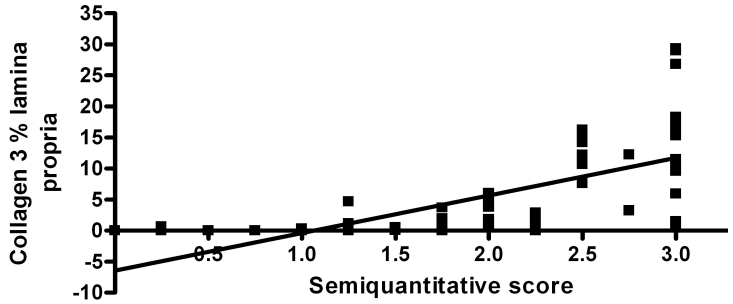


Table 2.2: Repeatability of collagen 3 analysis using semi quantitative and quantitative methods.

	Intra observer repeatability (number of repeat measurements)	**Inter observer repeatability (number of repeat measurements)r**
SQ-lamina propria	r_s=0.83 (n=12)	r_s=0.75 (n=68)
SQ-ASM	κ=0.47 (n=132)	κ=0.45 (n=132)
Q-lamina propria	r_s=1 (n=22)	r_s=1 (n=22)
Q-ASM	n/a	n/a

r_s= Spearman rank correlation coefficient.

κ= Cohen's Kappa[356] correlation coefficient [0-0.2; slight agreement, 0.21-0.40; fair agreement, **0.41-0.60; moderate agreement**, 0.60-0.80; substantial agreement, >0.80, excellent agreement].

2.2.3.4 Measurement of RBM thickening

RBM thickness was measured by a single observer at x400 magnification at 50 points orientated perpendicular to the epithelium separated by 20μm as previously described and validated by Sullivan et al[357]. The mean of these measurements was derived and quoted as the final RBM thickness.

The inter observer variability was determined by measuring RBM thickness by 2 observers (SS&CEB) in n=12 subjects and good agreement was found (r=0.79).

2.2.3.5 Determination of mast cell numbers in the airway smooth muscle.

We determined mast cell infiltration in the ASM in 2 sections at least 10 microns apart. The minimum area of ASM used to enumerate mast cells was 0.1mm^2 as previously described[105]. Tryptase positive cells within the ASM bundle were expressed/mm^2 of ASM and the mean of the 2 sections was used to determine the final count.

2.2.3.6 Quantification of Lamina Propria cell counts.

Nucleated immunostained cells staining for MBP (eosinophils), elastase (neutrophils) and tryptase (mast cells) were enumerated and expressed /mm^2 of the lamina propria. The mean cell count in 2 sections at least 20μm apart was used to determine the final count.

2.2.3.7 Quantification of vascular remodeling

2μm GMA sections were cut and stained using monoclonal antibodies for the pan endothelial marker EN4 (anti-PECAM-1/CD31) or appropriate isotype control. We used the chalkley count as a surrogate marker of vascular remodeling in the lamina propria. The chalkley count is surrogate for vascular density and area. The chalkley graticule is a circle with 25 randomly placed dots. The proportion of points lying over the image of one type of component (in this case a vessel with a visible lumen) is statistically

proportional to the area occupied by that component. The chalkley count has been used extensively to characterise the vascularity of tumours and has been shown to yield prognostic information in breast cancer and correlates closely with micro vascular density[358-360]. Furthermore the chalkley method is easy to perform, quick (3-4 minutes per count) and repeatable[358-360]. The chalkley eyepiece graticule was applied to 4 subjectively pre-determined, non-overlapping vascular hotspots in the lamina propria (1-2/section) at x200 magnification (corresponding to an area of 0.196mm^2) and rotated until the maximum number of vessels coincided with dots. All stained separate vessels with a lumen were included in the counts. The mean chalkley count (MCC) was derived. Chalkley counts were performed twice by a single observer blinded to clinical characteristics[358-360].

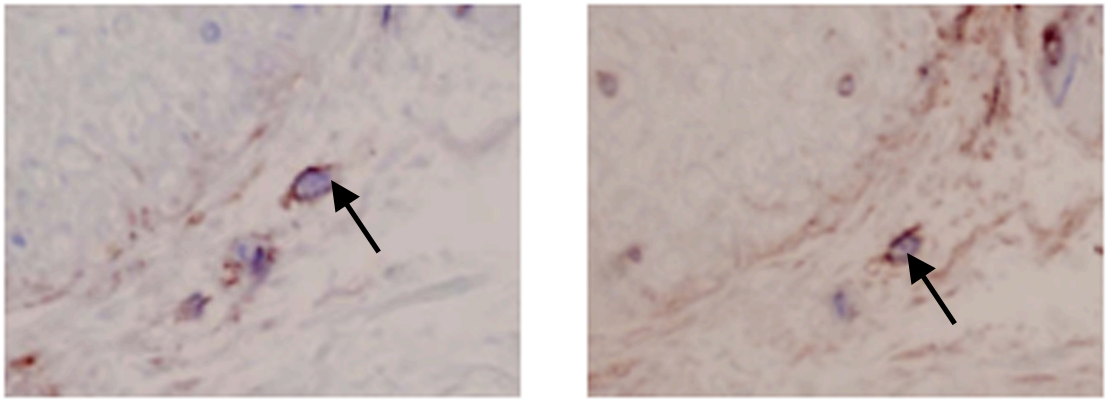
The mean chalkley count was found to be repeatable with a close correlation between blinded observer counts 2 weeks apart (Spearman r=0.83; p<0.0001; n=32).

2.2.3.8 Co localisation of fibrocytes

Sequential 2μm sections were cut and stained using monoclonal antibodies against CD34, collagen I, α-smooth muscle actin or appropriate isotype controls. Fibrocytes were identified as Collagen I$^+$ CD34$^+$ cells determined by the technique of co-localisation as described previously[142]. In brief, CD34+ cells were identified at high power (x400) within the lamina propria or ASM of the biopsy section. Images of the section were then taken at high resolution using the analysis system camera at (x400, x200 and x100) magnification. The sequential collagen 1 section was then photographed at (x400, x200 & x100 magnification) at the same orientation by rotating the camera while viewing the CD34+ section on the image analysis console. Composite images of collagen 1 and CD34+ sections for each magnification were then aligned side by side. Addition zoom factors were applied if necessary to confirm that individual cells were CD34 and Collagen 1 positive. The number of positive nucleated cells was enumerated per mm^2 of lamina propria or ASM-bundle by a blinded observer (figure 2.6). The minimum area of ASM used to enumerate fibrocytes in the ASM bundle was 0.1mm^2 as previously described[105].

Figure 2.6: Co localisation of fibrocytes in the airway wall

High power (x400 with a zoom factor of 50%) sequential 2μm sections stained for CD34 and collagen 1 in a patient with refractory asthma. The arrowed cell is a CD34+ Col-1+ fibrocyte adjacent to the airway smooth muscle bundle.



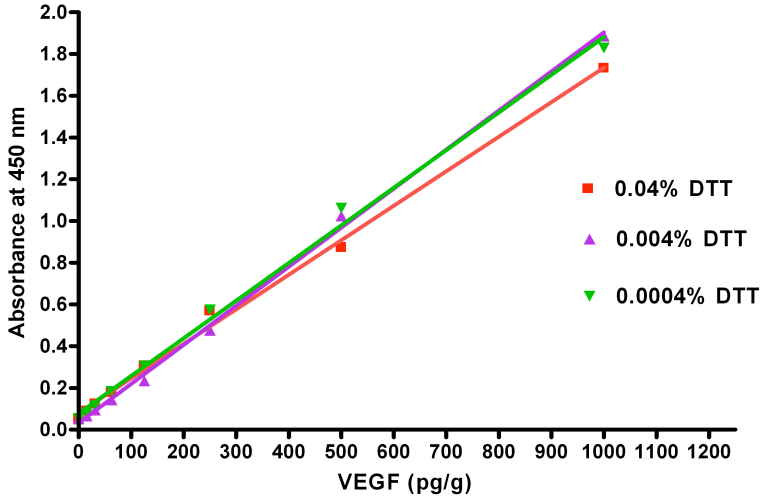
CD34 **Collagen 1**

2.2.4 Sputum Supernatant

2.2.4.1 Vascular Endothelial Growth Factor (VEGF) ELISA

Induced sputum supernatant VEGF was measured using a commercial ELISA (R&D Systems). Spiking experiments were performed throughout the range of the assay to assess recovery and VEGF standards were analysed to assess the effects of ditheiothreitol (DTT). Ditheiothreitol (DTT) did not significantly affect the VEGF standard curve (figure 2.7) and spiking experiments revealed a mean recovery [range] of 88% [84-93] (n=6). The minimum limit of detection was 7.8pg/ml. The inter/intra assay variability were 6.6 and 10% respectively.

Figure 2.7: The Effect of ditheiothreitol upon induced sputum supernatant VEGF standard curve.



3 Studies

3.1 Structural remodeling of the airway wall in asthma and EB.

3.1.1 Vascular Remodeling is a Feature of Asthma and Non Asthmatic Eosinophilic Bronchitis.

Abstract

Rationale: Increased vascularity and expression of vascular endothelial growth factor (VEGF) are recognised features of the asthmatic airway. The association of vascular remodeling with airway hyper-responsiveness (AHR) is unclear. **Objectives**: To assess vascular remodeling and sputum VEGF concentration in subjects with asthma, non-asthmatic eosinophilic bronchitis (EB) and healthy controls. **Methods:** *COHORT 1*- 19 asthmatics (GINA1-2 n=9, GINA 3-5 n=10), 10 patients with EB and 11 healthy matched controls were recruited. Expression of the endothelial marker EN4 was assessed in bronchial biopsy samples. Vessels were counted using the validated mean chalkley count (MCC) by a blinded observer. *COHORT 2*- A second independent cohort of 31 asthmatics (GINA1-2 n=11, GINA 3-5 n=20), 14 patients with EB and 15 matched controls were recruited. Induced sputum supernatant VEGF was measured by ELISA. **Results:** The MCC was significantly greater in GINA 3-5 asthma [5.2(0.4)] and EB [4.8(0.3)] compared to controls [3.5(0.5)] and demonstrated a significant inverse correlation with the post-bronchodilator FEV_1% predicted in asthmatics (R^2=0.28; p=0.02). Sputum VEGF concentration was also increased in GINA 3-5 asthma [2365(1361-4110)] pg/g and EB [4699(2818-7834)] pg/g compared to controls [1094(676-1774)] pg/g and was inversely related to post-bronchodilator FEV_1% predicted in asthma (R^2=0.2; p=0.01). **Conclusions:** Vascular remodeling is a feature of asthma and EB and is inversely associated with the post-bronchodilator FEV_1 in asthma suggesting that vascular remodeling is associated with airflow obstruction but not AHR.

Introduction

Asthma is characterised by symptoms, variable airflow obstruction, airway hyper-responsiveness (AHR) and inflammation. In addition there are a number of changes to the structural components of the airway wall collectively known as remodeling[14;284]. Vascular remodeling and increased expression of associated growth factors such as basic-fibroblast growth factor and vascular endothelial growth factor (VEGF) are well-recognised features of asthma[59;90;164-167;361;362]. This vascular remodeling is likely to be functionally important as enhanced vascularity in the inner wall of the medium airways correlates with airflow obstruction in asthma[89]. However, the relationship between vascular remodeling and AHR is uncertain.

Non-asthmatic eosinophilic bronchitis (EB) is an airway disease that has emerged from the study of chronic cough and airway inflammation and accounts for approximately 10% of referrals to a specialist cough clinic[310]. EB is characterised by eosinophilic airway inflammation in sputum, bronchoalveolar lavage and bronchial biopsy, without any evidence of variable airflow obstruction or AHR[310;314]. The EB phenotype remains stable over time with few patients developing asthma; however, some patients develop fixed airflow obstruction, particularly if eosinophilic airway inflammation is severe and not controlled by inhaled corticosteroids[312]. EB is therefore a powerful disease control model to study potential mechanisms of AHR. Importantly over expression of IL-13 by mast cells and eosinophils in the bronchial submucosa[316] and peripheral blood[363] and mast cell co-localisation to airway smooth muscle[105] are immunopathological features of asthma that are not shared by EB and have therefore been implicated in the pathogenesis of AHR. In addition, Kanazawa et al demonstrated that VEGF was increased in the sputum in asthma but not in EB and correlated with increased airway vascular permeability[62]. VEGF levels in sputum and airway vascular permeability showed significant inverse correlations with AHR in asthma, suggesting that vascular remodeling and airway wall oedema are important causes of AHR in asthma. However the selection of patients with chronic disease is important in the study of airway remodeling[319] and the duration of disease was unclear in the study by Kanazawa et al.

Therefore whether vascular remodeling is a feature shared by asthma and EB or is indeed a characteristic unique to asthma needs to be clarified.

We sought to assess the relationship of vascular remodeling and induced sputum VEGF with AHR, airflow obstruction by comparison of asthma with EB in patients with chronic disease.

Methods

Subjects

Subjects were recruited from Glenfield Hospital outpatients, staff and by local advertising. All subjects were non-smokers with a smoking history of <10 pack years; had been free of exacerbations and on stable treatment for 8 weeks prior to entry into the study. EB was defined according to the American College Of Chest Physicians ACCP criteria[364]. In brief, patients had a history of chronic cough, without symptoms of variable airflow obstruction, a sputum eosinophil count of > 3% and normal lower airway responsiveness to methacholine (PC_{20}>16mg/ml). Asthma was defined and severity classified using the current GINA guidelines[10] based upon the GINA treatment steps (GINA 1-2 Intermittent to mild persistent asthma; GINA 3-5 moderate to severe persistent asthma) and the American Thoracic Society (ATS) criteria for Refractory asthma[9]. Normal subjects had no history of respiratory disease and normal spirometry. The Leicestershire ethics committee approved the study and all patients gave their written informed consent.

Two independent cohorts (tables 3.1&3.2) were recruited to assess vascular remodeling in bronchial biopsies (cohort 1: 19 subjects with asthma, 10 with EB and 11 controls) and VEGF in sputum (cohort 2: 31 subjects with asthma, 14 with EB and 15 controls). 9/24 of the subjects with EB, 8/50 of the subjects with asthma and 11/26 controls had taken part in previous studies comparing the immunopathology of asthma and EB[105;314].

Protocol and clinical characterisation

All subjects underwent spirometry, allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat and grass pollen, measurement of exhaled nitric oxide (eNO) concentration (measured at 50mls/s NIOX; Aerocrine, Stockholm, Sweden), a methacholine inhalation test[241] and sputum induction[210]. One week later, patients in Cohort 1 underwent bronchoscopy conducted according to the British Thoracic Society guidelines[352]. Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae and were fixed in acetone and embedded in glycomethacrylate (GMA)[353].

Immunohistochemistry & Chalkley counts

Cohort 1

2μm GMA sections were cut and stained using monoclonal antibodies for EN4 (anti-PECAM-1/CD31; Monosan; Uden, Netherlands), major basic protein MBP (Caltag, Paiseley, UK) or appropriate isotype control.

Quantification of airway structure and cellular localisation.

Morphometry was assessed using computer-assisted image analysis. RBM thickening (in μm) was assessed using the 50-point method as previously described[105;357]. Nucleated immunostained cells were enumerated in the lamina propria and expressed as cells /mm^2 of the lamina propria (see methods section).

Chalkley counts were performed twice by a single observer blinded to clinical characteristics[358-360].The chalkley count is surrogate for vascular density and area. The chalkley graticule is a circle with 25 randomly placed dots. The chalkley eyepiece graticule was applied to 4 subjectively pre-determined, non-overlapping vascular hotspots in the lamina propria (1-2/section) at x200 magnification (corresponding to an area of 0.196mm^2) and rotated until the maximum number of vessels coincided with dots. All stained separate vessels with a lumen were included in the counts. The mean chalkley count (MCC) was derived.

Sputum VEGF.

Cohort 2

Induced sputum supernatant VEGF was measured using a commercial Enzyme-Linked ImmunoSorbent assay ELISA (R&D Systems, Abingdon, United Kingdom).

Table 3.1: Clinical characteristics Cohort 1

	Control ***(n=11)***	**Eosinophilic Bronchitis** ***(n=10)***	**Asthma (n=19)** **GINA 1-2 n=9** **GINA 3-5 n=10** **(Refractory asthma n=5)**
Age (yrs)	36(6.1)	45.8(4.1)	46.9(3.9)
Sex M:F	7:4	6:4	6:13
Atopy (%)	27	50	58
Disease duration (yrs)	0	5.5(1.6)	10.3(2.7)
Inhaled BDP (g/24hrs)	0	0	821(177)^^
Oral prednisolone	0	0	4/19
Pre Bronchodilator FEV_1 (l)	3.53(0.25)	2.86(0.23)	2.1(0.22)*¶
Post Bronchodilator FEV_1 (l)	3.55(0.25)	2.89(0.24)	2.45(0.20)*¶
Post Bronchodilator FEV_1 %	100(4.3)	94(3.5)	91(4.0)
Post Bronchodilator FEV_1/FVC	81(2.1)	79.5(2.5)	76(3.0)
PC_{20} (mg/ml)#	>16	>16	0.86[0.03-4.6]*^^
ENO (ppb) #	7.3 [5.5-9.6]	19.5 [13.6-28.0]	23[15.7-33.4]*ψ
Induced sputum			
Eosinophils (%) #	0.45 [0.2-0.9]	7.0 [4.5-10.9]	4.0 [1.5-10.3]*ψ
Neutrophils (%) #	31[17.4-55.2]	45.9 [28.5-73.8]	35.7 [22.0-57.5]
Lamina propria eosinophils/mm^2 **	9.9[5.3-20.5]	30.2 [15.5-71.4]	14.9[5-25]
Reticular basement membrane thickness (m)	7.35 (0.42)	12.0 (1.41)	11.20(0.84)*ψ

*Data expressed as mean (SEM); #Geometric mean [95%CI], ** Median [interquartile range]. BDP equivalents; fluticasone 2:1, Budesonide 1.25:1, Mometasone 1.25:1. Intergroup comparisons: t-test and one-way ANOVA with Bonferroni correction for multiple comparisons. * $p<0.05$ ANOVA; ^^ $p<0.05$ asthma vs. EB, ¶ $p<0.05$ asthma vs. control. ψ $p<0.05$ EB/Asthma vs. control.*

Table 3.2: Clinical characteristics Cohort 2

	Control *(n=15)*	**Eosinophilic Bronchitis *(n=14)***	**Asthma (n=31) GINA 1-2 n=11 GINA 3-5 n=20 (Refractory asthma n=12)**
Age (yrs)	36.5 (4.3)	56.6 (3.7)	45.3 (2.7)*
Sex M:F	7:8	10:4	19:12
Atopy (%)	33	50	66^
Disease duration (yrs)	N/A	7.8(1.4)	18.2 (14.6)^^
Inhaled BDP (g/24hrs)	N/A	1043(236)	1342 (224)
Oral prednisolone	N/A	0	8/31
Intramuscular Triamcinolone	N/A	0	4/31
Pre Bronchodilator FEV_1(l)	3.53(0.27)	2.82(0.19)	2.47(0.17)*¶
Post Bronchodilator FEV_1(l)	3.52(0.28)	2.92(0.19)	2.65(0.15)*¶
Post Bronchodilator FEV_1 %	100(4.3)	94(3.5)	91(4.0)
Post Bronchodilator FEV_1/FVC	84.6 (3.1)	78.8 (2.5)	74.5 (1.9)*
PC_{20} (mg/ml)#	>16	>16	1.06 [0.39-2.93]*^^
ENO (ppb) #	14.0 [9.9-19.8]	26.1 [15.8-43.1]	30.3 [20.1-45.8]
Induced sputum			
Eosinophils (%) #	0.53 (0.25-1.2)	3.3 (1.2-9.2)	3.0 (1.4-6.5)*ψ
Neutrophils (%) #	43.3 (26-72)	44.8 (21-65)	36.5 (20-65)

Data expressed as mean (SEM); #Geometric mean [95%CI]

*Intergroup comparisons: t-test and one-way ANOVA with Bonferroni correction for multiple comparisons. BDP equivalents; fluticasone 2:1, Budesonide 1.25:1, Mometasone 1.25:1 ^ $p<0.05$ χ^2 test, * $p<0.05$ ANOVA; ^^ $p<0.05$ asthma vs. EB, ¶ $p<0.05$ asthma vs. Control, ψ $p<0.05$ EB/Asthma vs. control.*

Analysis

Statistical analysis was performed using PRISM Version 4 (Prism, San Diego, Calif). Parametric data were expressed as mean (SEM), data that had a log normal distribution was log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (range). One-way analysis of variance and Student's t-tests were used for across and between group comparisons respectively. Chi squared tests were used to compare categorical data. We chose to examine the relationship between disordered airway physiology (post-bronchodilator FEV_1 and AHR) and vascular remodeling by univariate analysis Pearson and Spearman rank correlation coefficients as appropriate. Stepwise linear regression was performed to assess which variables best correlated with vascular remodeling in each cohort. Independent variables entered into the regression model were age, daily equivalent dose of inhaled beclomethasone diproprionate, duration of disease, log transformed % sputum eosinophil count and eNO, lamina propria eosinophil count (cohort 1), RBM thickness (Cohort 1), FEV_1 % predicted and log transformed methacholine PC_{20}. Regression data are presented as model adjusted Pearson correlations along side the standardised correlation coefficient (β) of the modelled independent variable. A p value of <0.05 was taken as the threshold for statistical significance.

Results

Vascular remodeling is a feature of moderate and severe persistent asthma, refractory asthma and EB

Examples of immunohistochemical staining are shown in figure 3.1 a-d. The mean chalkley count was found to be repeatable with a close correlation between blinded observer counts 2 weeks apart (Spearman $r=0.83$; $p<0.0001$; $n=32$).

The mean chalkley count was significantly elevated in patients with moderate to severe persistent asthma (GINA3-5) [5.2(0.4)] p=0.02, refractory asthma alone [4.8(0.3)] p=0.04 and EB [5.1(0.20)] p=0.01; in comparison to the control group [3.5(0.5)] (figure 3.2). There was no significant difference between the MCC in refractory asthma

compared to patients with GINA 1-2 asthma [4.1(0.54)] p=0.33 and GINA 3-5 (non-refractory) asthma [5.6(0.70)] p=0.37.

Increase sputum VEGF is a feature of moderate and severe persistent asthma, refractory asthma and EB

The median (range) induced sputum VEGF concentration was significantly elevated in subjects with moderate to severe persistent asthma (GINA 3-5) 2.4 (1.4-4.1) ng/ml (p=0.04), refractory asthma 3.7 (1.8-7.5) ng/ml (p=0.004) and EB 4.7 (2.8-7.8) ng/ml (p=0.0001) compared to the healthy controls 1.1 (0.7-1.8) ng/ml (figure 3.3).

Vascular remodeling is associated with post-bronchodilator lung function and disease duration in asthma.

Univariate analysis revealed a significant inverse correlation between the post-bronchodilator FEV_1 % predicted in the subjects with asthma and the MCC (R^2=0.28; p=0.02) (figure 3.4a) and log VEGF sputum concentration (R^2=0.21; p=0.01) (figure 3.4b). There was no significant relationship between post-bronchodilator FEV_1 % predicted in the subjects with EB and MCC (R^2=0.02; p=0.72) or sputum VEGF concentration (R^2=0.02; p=0.64).We did not find any association between the log transformed methacholine PC_{20} and the MCC [R^2=0.04(p=0.46)], or sputum VEGF concentration [R^2=0.013(p=0.7)] in asthmatic subjects.

Step-wise linear regression demonstrated that post- bronchodilator FEV_1 % predicted in combination with the lamina propria eosinophil count best modelled the MCC in asthma (Model adjusted R^2=0.43; β= -0.46 (Post-bronchodilator FEV_1 % predicted) p<0.0001; β= -0.42 (lamina propria eosinophils) p=0.001). Disease duration was the best predictor of sputum VEGF in asthma (Model adjusted R^2=0.24; β=0.50 p<0.0001).

Figure 3.1: Immunohistochemistry vascular remodeling

Representative photomicrographs (x200) of bronchial biopsy sections stained with monoclonal antibody against PECAM-1/EN4. a) Control subject illustrating relatively few vessels, b) increased vascularity in a mild intermittent asthmatic (GINA 1), c) non-asthmatic eosinophilic bronchitis (EB) subject, multiple vessels in the lamina propria, and d) refractory asthma subject, multiple vessels surrounding submucosal glands. RBM thickening is evident in asthmatic and EB subject.

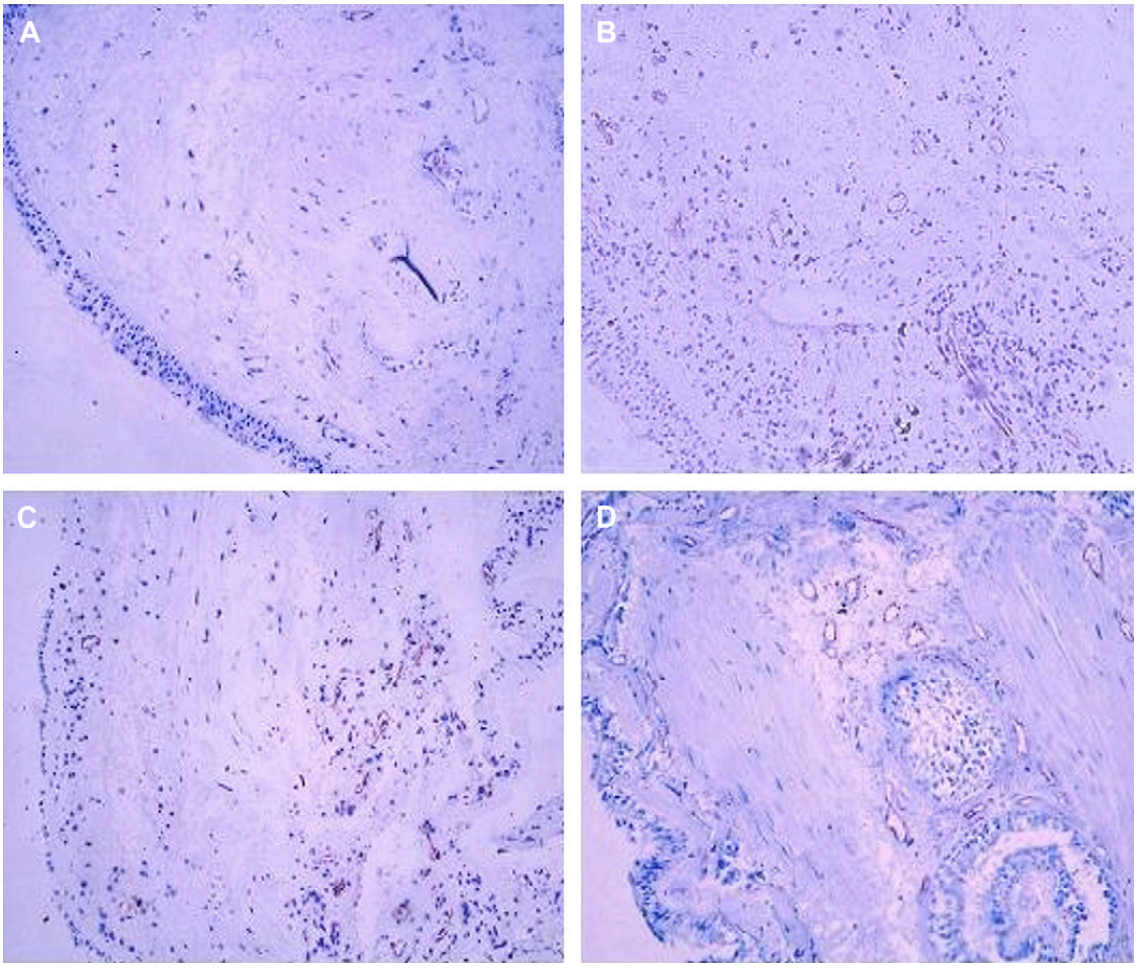


Figure 3.2: Mean chalkley counts Cohort 1

The mean chalkley count in the control group ■ , GINA 1-2 (intermittent to mild persistent asthma) ▲; GINA 3-5 (moderate to severe persistent asthma) including refractory asthma alone ◆ and non-refractory ▼; and non-asthmatic eosinophilic bronchitis (EB) ●.

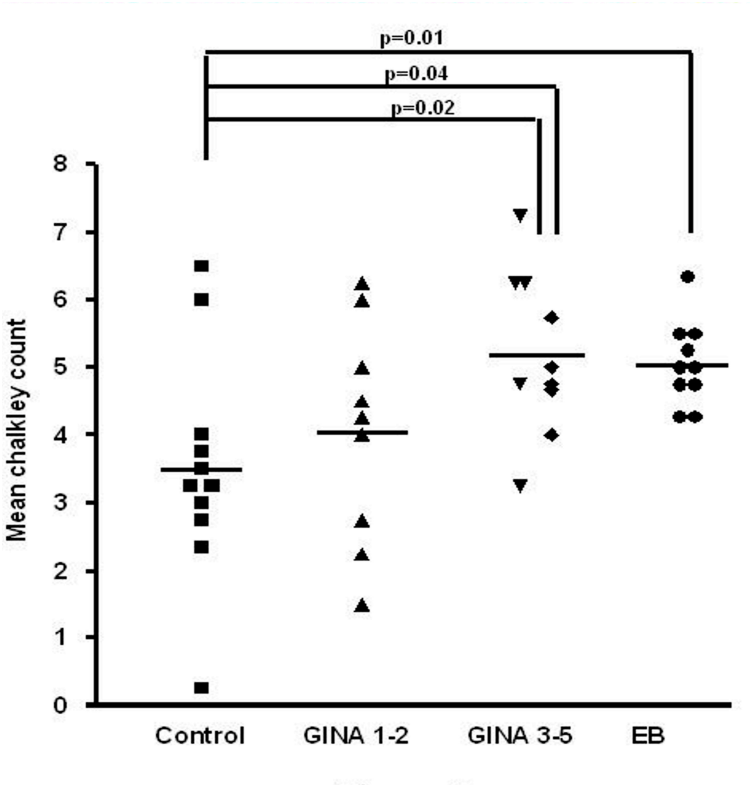


Figure 3.3: Induced sputum supernatant VEGF (pg/g); Cohort 2.

Sputum VEGF concentration in the control group ■ ; GINA 1-2 (intermittent to mild persistent asthma) ▲; GINA 3-5 (moderate to severe persistent asthma) including refractory asthma alone ◆ and non-refractory ▼; and non-asthmatic eosinophilic bronchitis (EB) ●.

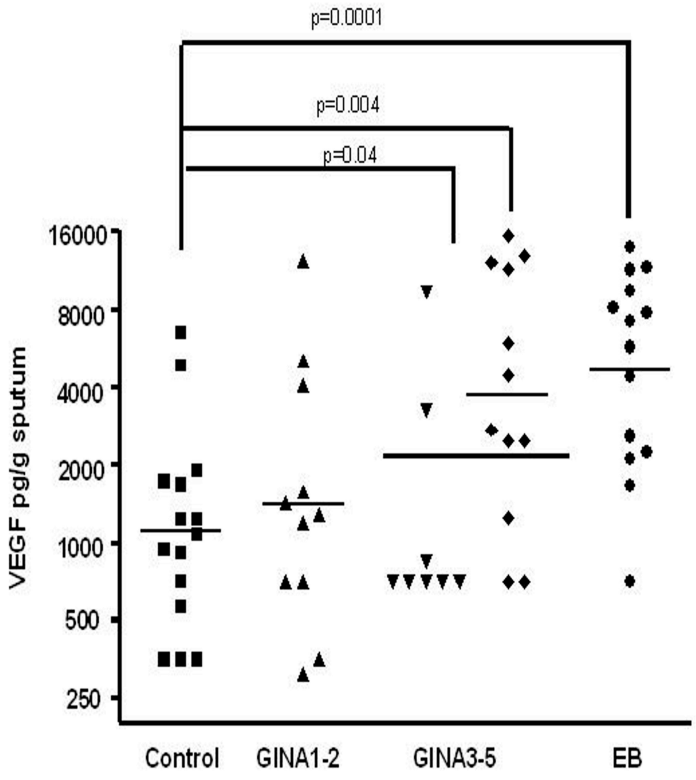
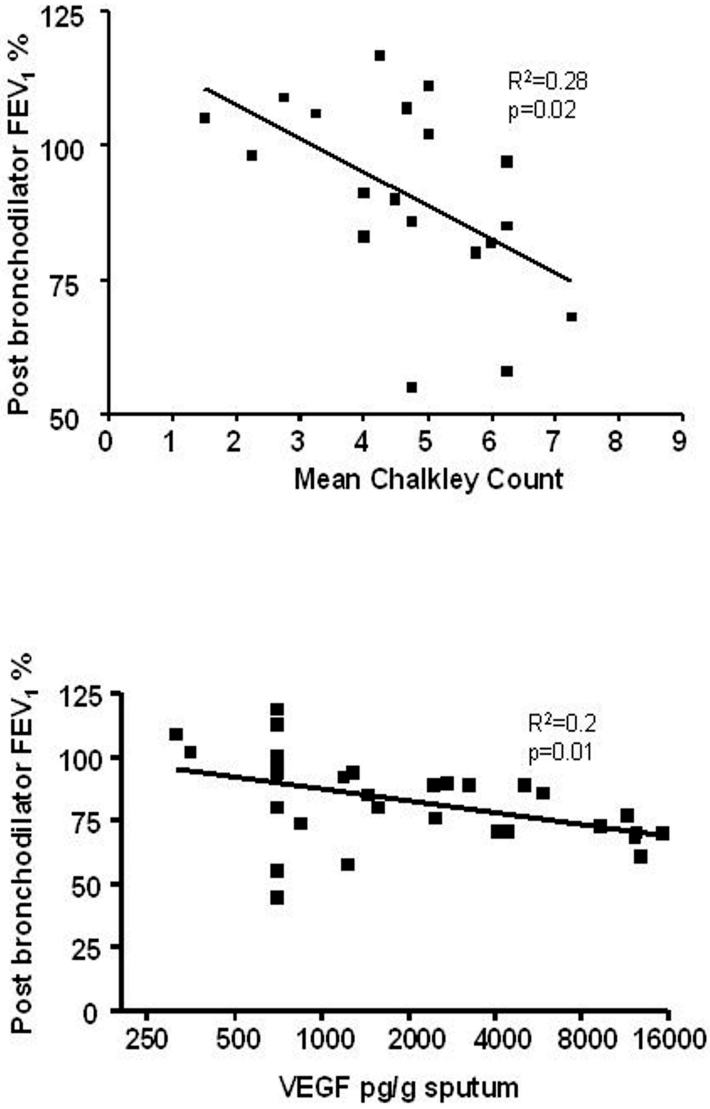


Figure 3.4: Vascular remodeling and airflow obstruction in asthma



Discussion

We have shown for the first time that sputum VEGF concentration and vascularity in the bronchial lamina propria were increased in non-asthmatic eosinophilic bronchitis (EB) to a similar intensity as observed in moderate to severe persistent and refractory asthma. Our assessment of sputum VEGF and vascular remodeling in bronchial biopsies were made in two independent cohorts and the consistency of our findings in each cohort strengthens our findings. The presence of vascular remodeling in EB demonstrates that vascular remodeling and AHR can be dissociated and therefore questions its role in the pathogenesis of AHR in asthma.

Our findings indicate that vascular remodeling is a feature of EB. This is in contrast to the findings of an earlier study by Kanazawa et al that demonstrated that increased sputum VEGF and vascular permeability were not features of EB[62]. One possible explanation for the discrepancy between these studies is disease duration. We carefully selected only patients that had EB for at least 1 year and the median length of disease was over 5 years in both cohorts. Interestingly the view that sputum VEGF and disease duration are associated finds support from our finding that using step wise linear regression disease duration was the strongest predictor of sputum VEGF in asthma. In addition we have confirmed our findings in two independent cohorts of patients using different methods to assess vascular remodeling. Therefore we are confident that our findings are robust. The view that AHR and features of remodeling are independent is consistent with several other lines of evidence. We report here, and in an earlier study[105], that thickening of the lamina reticularis and basement membrane (RBM) is a feature shared by EB and asthma and was not related to AHR. Indeed, Milanese et al have demonstrated a positive relationship between RBM thickening and the dose of methacholine to cause a 20% fall in FEV_1 in asthma suggesting that remodeling may protect against AHR[365]. Similarly, Ward et al demonstrated that δVd the change in anatomical dead space with lung volume, which has been proposed as an index of airway distensibility or stiffness correlates inversely with RBM thickening in asthma[128]. It is important to note that the measurement of RBM thickening alone is not an overall assessment of airway wall remodeling. However, using measurements of airway wall

geometry derived from HRCT images, airway wall thickening was inversely related to airway reactivity[67]. Thus, taken together, these findings support the view that airway remodeling in contrast to contributing to AHR may indeed protect against AHR.

To date comparisons of EB and asthma have been very informative about the potential mechanisms of AHR. The cardinal immunopathological feature that distinguishes EB and asthma is the presence of mast cells within the ASM-bundle in asthma[105]. Several studies have confirmed that mast cell numbers in the ASM-bundle are increased[43;77;110;111] and the intimacy of these cell types is illustrated by studies using electron microscopy[113]. Importantly the number of mast cells in the ASM-bundle is related to the degree of the AHR[105]. The mast cells are activated in the ASM-bundle with increased degranulation[112] and they express the cytokines IL-4 and IL-13[366]. Importantly there is evidence to suggest that the ASM phenotype in asthma is altered[142;202] and it is likely that these intrinsic abnormalities in ASM function together with interactions with mast cells rather than airway remodeling that are the key determinants of AHR.

The lack of association between vascular remodeling and AHR does not exclude the possibility that vascular remodeling is important in the development of other aspects of disordered airway physiology in asthma. We found that the bronchial lamina propria vascularity and sputum VEGF concentration correlated inversely with post-bronchodilator lung function in asthma suggesting that vascular remodeling contributes to persistent airflow obstruction. This is entirely consistent with a previous report demonstrating an association between vascular remodeling and airflow obstruction in asthma[89]. We and others have demonstrated that fixed airflow obstruction may develop in EB[312;367] particularly in those subjects with poorly controlled eosinophilic inflammation. However, we did not observe an association between lamina propria vascularity or sputum VEGF concentration and post-bronchodilator lung function in the subjects with EB. Our subjects with EB had normal lung function and some in *Cohort 2* were treated with inhaled corticosteroids leading to a reduction in the intensity of their eosinophilic inflammation. Both of these factors are likely to have reduced the chance

of observing a relationship between lung function and vascular remodeling in this group. Therefore, the relative contribution of vascular remodeling to the development of airflow obstruction in some patients with EB requires further study.

The focus of our study was the role of vascular remodeling in the development of the disordered airway physiology in asthma. We have therefore not undertaken an extensive investigation of other features of remodeling such as glandular hyperplasia, matrix deposition or ASM hyperplasia and hypertrophy. Importantly, others have demonstrated that increased ASM mass was associated with severity of airflow obstruction in asthma[76] and therefore there is a need to extend our observations in later studies to include several features of airway remodeling. One possible weakness of our study was the cross-sectional design. There is a paucity of longitudinal studies investigating the relationship between remodelling and disordered airway physiology. We have attempted to partly address this shortcoming by including subjects with chronic disease and the inclusion of EB as a disease control group strengthens our assertions that vascular remodeling was not associated with AHR. Intriguingly, the lamina propria eosinophil count was inversely related to vascular remodeling in asthma as assessed by the MCC. The cross-sectional design of this study means that we do not have a measure that reflects eosinophilic inflammation over time. A possible explanation for this apparent anomaly is that subjects with increased vascular remodeling are those subjects that had persistent eosinophilic inflammation, which is now controlled by corticosteroid therapy. Future studies need to examine longitudinally the natural history of airway remodeling, its relationship to airway function, airway inflammation and response to treatment.

In conclusion we have shown for the first time that vascular remodeling and increased expression of VEGF are features of non-asthmatic eosinophilic bronchitis and moderate to severe persistent and refractory asthma; and are dissociated from AHR but are associated with airflow limitation. Our work provides further evidence of the dissociation of airway remodeling and AHR in asthma.

3.1.2 Airway hyperresponsiveness is dissociated from airway wall structural remodeling.

Abstract

Background. Non asthmatic eosinophilic bronchitis (EB) has emerged as a powerful disease control model to study the structural and inflammatory mechanisms of airway hyperresponsiveness (AHR) in asthma. We have previously shown that vascular remodeling and reticular basement membrane (RBM) thickening are present in EB. However, it is not known whether other features of structural remodeling including increased airway smooth muscle (ASM) mass, matrix deposition and glandular hyperplasia are also present in EB. **Objectives.** We sought to determine whether structural remodeling occurs in EB and is associated with AHR and airflow limitation. **Methods.** 42 asthmatics, 21 patients with EB and 19 healthy volunteers were recruited. ASM area, RBM thickness, collagen 3 deposition, glandular area, mast cells and granulocytes were assessed in bronchial biopsy samples. **Results.** EB and asthma were associated with a significant increase in ASM mass and RBM thickness compared to healthy subjects. In contrast we did not observe any significant differences in collagen 3 deposition in the lamina propria and ASM or the % of submucosal glands. Univariate analysis demonstrated that mast cell numbers in the ASM was the only feature of remodeling that was associated with AHR (β=-0.51; p=0.004). Stepwise linear regression revealed that a combination of mast cell numbers in the ASM [β= -0.43] and disease duration [β= -0.25] model adjusted R^2=0.26; p=0.027; best modelled AHR. **Conclusion.** Mast cell localisation to the ASM bundle, but not structural remodeling of the airway wall, is associated with AHR in asthma.

Introduction

Asthma is a common airway disease that accounts for significant healthcare cost[330]. It is characterised by variable airflow limitation, airway inflammation and airway hyper responsiveness (AHR). Structural and cellular changes within the airway wall in asthma, notably increased airway smooth muscle (ASM) mass[79], vascular remodeling[368], thickening of the reticular laminar and basement membrane (RBM) and fibroblast numbers in the subepithelium[76], have been shown to correlate with airflow limitation. Furthermore cellular infiltration of the airway wall in asthma is related to decline in lung function[350].The association of structural change in the airway wall in asthma with AHR is much more tenuous. A number of reports have drawn conflicting conclusions of the association of AHR with epithelial desquamation/loss of tight junctions[369-371], RBM thickening[80;95;193;372;373], vascular remodeling[362;368] and ASM mass[79;88].

Non asthmatic eosinophilic bronchitis (EB) has emerged as a powerful disease control model to study the mechanisms of AHR in asthma[374]. EB is a common cause of chronic cough accounting for approximately 10% of referral to a specialist cough clinic[310] and is characterised by eosinophilic airway inflammation. However in contrast to asthma there is an absence of variable airflow obstruction and AHR[364]. We have previously shown that EB is characterised by RBM thickening[314] and vascular remodeling[368] to a similar degree as patients with asthma and eosinophilic airway inflammation. In contrast mast cell infiltration of the ASM bundle was a defining feature of the asthma phenotype being absent in EB and healthy subjects[105]. Importantly, studies examining the natural history of EB have demonstrated that fixed airflow obstruction may occur[312] , which would again support the notion that structural remodeling of the airway wall may occur in EB.

We therefore hypothesised that 1) structural remodeling of the airway wall does occur in EB, 2) structural remodeling of the airway wall is dissociated from AHR in asthma and 3) mast cell localisation to the ASM-bundle is a key determinant of AHR in asthma.

Methods

Subjects

21 subjects with EB, 42 with asthma, and 19 normal controls were recruited from Glenfield Hospital outpatients, staff, and by local advertising.

Asthma was defined by one or more of the following objective criteria; significant bronchodilator reversibility of >200mls, a provocation concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) of less than 8mg/ml or a peak flow amplitude % mean over 2 weeks of more than 20%. Asthma severity was classified using the current GINA guidelines based upon the GINA treatment steps[10]; GINA 1-2 (n=17) intermittent to mild persistent asthma; GINA 3-4 (n=15) moderate to severe persistent asthma and the American Thoracic Society (ATS) criteria for refractory asthma (n=10)[9]. Normal subjects had no history of respiratory disease and normal spirometry and methacholine responsiveness. Non-asthmatic eosinophilic bronchitis (EB) was defined by the American College of Chest Physicians Criteria[364]. In brief, patients had a history of chronic cough, without symptoms of variable airflow obstruction, a sputum eosinophil count of > 3% and normal lower airway responsiveness to methacholine (PC_{20}>16mg/ml). All subjects were non-smokers with a past smoking history of less than 10 pack years.

12 patients with EB, 12 asthmatics and 7 healthy controls were included in previous studies[314;375]. The Leicestershire ethics committee approved the study and all patients gave their written informed consent.

Protocol and clinical measurements

Subjects attended on two occasions. At the first visit exhaled nitric oxide [(eNO) measured at 50mls/s NIOX; Aerocrine, Stockholm, Sweden], spirometric parameters before and after bronchodilator (400μg inhaled albuterol), allergen skin prick tests and methacholine airway responsiveness using the tidal breathing method (0.03 to 16 mg/ml)[241] were measured, followed on recovery by sputum induction[210]. At the second visit 1 week later the subjects underwent bronchoscopy[352]. Mucosal biopsy specimens were processed into the water soluble resin glycol methacrylate (GMA) (Polysciences, Northampton, UK) for embedding[353].

Immunohistochemistry

Two μm sections were cut, floated on 0.2% ammonia solution in water for 1 min, and dried at room temperature for 1–4 hrs. The following mouse IgG_1 monoclonal antibodies were used α-smooth muscle actin (Dako, Cambridge, UK), Collagen type 3 (Sigma, Gillingham, UK), tryptase (Dako, Cambridge, UK), major basic protein (Caltag, Paiseley, UK) and neutrophil elastase (Dako, Cambridge, UK) with appropriate isotype controls (Dako, Cambridge, UK). The technique of immunostaining applied to GMA embedded tissue has been described previously[353].

Quantification of airway structure and cellular localisation.

Morphometry was assessed using computer-assisted image analysis. The percentage (%) of the bronchial lamina propria occupied by ASM, glands and collagen 3 was determined. RBM thickening (in μm) was assessed using the 50-point method as previously described[357]. Nucleated immunostained cells were enumerated in the lamina propria/ASM and expressed as cells /mm^2 of the lamina propria and ASM (see methods section).

Table 3.3: Baseline clinical characteristics

	Control *(n=19)*	**Eosinophilic Bronchitis *(n=21)***	**Asthma *(n=42)***		
			GINA 1 (n=17)	**GINA 2-4 (n=15)**	**Refractory (n=10)**
Age (yrs)	36.4(3.2)	49.8(2.6)*	45.4(3.9)	52.9(4.4)*	47.1(2.1)¶
Sex M:F	8:11	10:11	7:10	9:6	4:6
Atopy (%)	44.4	53.3	75	70	50$^{\infty}$
Disease duration (yrs)	na	5.0(1.3)	12.0(2.5)	15.9(3.9)^	18.0(5.1)¶^
Inhaled BDP (g/24hrs)	na	200(84)	0	1067(122)^^	1404(179)¶^^
Oral prednisolone [number/dose)	na	0/21;0	0/10;0	0/15;0	8/10; 12.2(3.2)
Post Bronchodilator FEV_1 (l)	3.0(0.16)	3.8(0.26)	2.9(0.28)	2.8(0.2)^	2.5(0.25)¶^
Post Bronchodilator FEV_1 %	105.5(2.6)	99.6(2.9)	96.3(4.0)	94.694.6)	80.7(6.5)¶^*
FEV_1/FVC post bronchodilator	85.0(2.7)	81.4(1.2)	75.4(2.8)	76.5(2.0)	70.2(2.8)¶^*
PC_{20} (mg/ml)#	>16	>16	0.62[0.25-1.5]	0.44[0.17-1.1]	0.35[0.02-5.5]¶

*Data expressed as mean (SEM); #Geometric mean [95%CI], ** Median [interquartile range]. BDP equivalents (Beclamethasone Diproprionate/24 hrs); fluticasone 2:1, Budesonide 1.25:1, Mometasone 1.25:1.*

¶ $p<0.05$ one-way ANOVA ; Normal vs. EB/GINA1, GINA2-4, Refractory asthma.

**$p<0.05$ vs. control (Bonferroni /Dunns Correction for multiple comparisons)*

^ $p<0.05$ vs. EB (Bonferroni /Dunns Correction for multiple comparisons)

^^ $p<0.05$ vs. EB & GINA 1 asthma (Bonferroni /Dunns Correction for multiple comparisons)

$\infty p<0.0001$ χ^2 squared test

Table 3.4: Baseline Inflammatory characteristics.

	Control *(n=19)*	**Eosinophilic Bronchitis *(n=21)***	**Asthma *(n=42)***		
			GINA 1 (n=17)	**GINA 2-4 (n=15)**	**Refractory (n=10)**
Induced Sputum					
Eosinophils (%) #	0.33[0.25-0.43]	6.9[3.9-12]*	2.0[0.7-5.2]*	1.7[0.66-4.6]*	6.9[1.2-38.5]¶
Neutrophils (%)	52.9(10.4)	53.4(5.5)	45.6(7.3)	55.0(8.3)	44.9(10.0)
Lamina propria					
Mast Cells/mm^2**	15.9[10.1-21.7]	28.1[16.0-35.6]	19.5[15.7-25.3]	19.0[11.2-28.0]	17.0[13.2-29.8]¶
Eosinophills/mm^2**	2.3[1.1-5.4]	31.7[18.3-57.7]*	20.9[7.8-38.8]*	11.3[4.9-19.7]	28.1[6.6-31.9]¶*
Neutrophils/mm^2**	7.7[2.1-10.3]	35.5[16.8-46]*	15.4[10.6-27.2]	19.5[5.4-40.7]	15.6[3.2-22.3]¶

*Data expressed as mean (SEM); #Geometric mean [95%CI], ** Median [interquartile range].*

¶ $p<0.05$ one-way ANOVA ; Normal vs. EB/GINA1, GINA2-4, Refractory asthma.

**$p<0.05$ vs. control (Bonferroni /Dunns Correction for multiple comparisons)*

Analysis

Statistical analysis was performed using PRISM Version 4 (Prism, San Diego, Calif) and regression analysis using SPSS Version 13.0 (SPSS Inc, Chicago III). Parametric data were expressed as mean (SEM), data that had a log normal distribution was log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (range). One-way analysis of variance and Student's t-tests were used for across and between group comparisons respectively. Chi squared tests were used to compare categorical data. We chose to examine the relationship between disordered airway physiology (post-bronchodilator FEV_1 and AHR) and structural remodeling by univariate analysis pearson correlation coefficients. Stepwise linear regression was performed to assess which variables best correlated with airway hyper responsiveness in asthma. Independent variables entered into the regression model were age of onset, daily equivalent dose of inhaled beclomethasone diproprionate, duration of disease, log transformed % sputum/lamina propria eosinophil count, % sputum/lamina propria neutrophil count, RBM thickness, % (ASM, glands, in the lamina propria), % area of lamina propria matrix, mast cell numbers/mm^2 of ASM and dependant variable of log transformed methacholine PC_{20}.

Results

Airway structural remodeling is present in both eosinophilic bronchitis and asthma.

EB and asthma were associated with a significant increase in ASM mass and RBM thickness compared to healthy subjects. ***Table 3.5 & Figures 3.5a&b***. In contrast we did not observe any significant differences in lamina propria or ASM collagen 3 deposition ***Figure 3.6a&b***. We observed a trend for increased % glands in the lamina propria in refractory asthma (p=0.08). ***Table 3.5***.

Table 3.5: Airway structural changes in Asthma and EB.

	Control ***(n=19)***	**Eosinophilic Bronchitis** ***(n=21)***	**Asthma** ***(n=35)***		
			GINA 1 (n=17)	**GINA 2-4 (n=15)**	**Refractory (n=10)**
RBM thickness (µm)	6.6(0.4)	11.1(0.96)*	9.5(0.77)*	8.9(1.1)	11.0(0.7)¶*
% ASM	8.5(1.3)	17.9(3.4)*	17.2(1.9)*	16.1(3.6)	16.3(2.8)¶*
% Glands	1.6(0.61)	3.1(1.4)	0.82(0.3)	2.4 (1.5)	4.9(1.6)
Collagen 3 % LP	8.9(2.3)**	7.4(2.1)**	2.6(1.3)	1.4(0.4)	6.9(2.3)¶**
Collagen 3 SQS LP	2.02(0.2)	2.36(0.15)	1.85(0.27)	1.68(0.16)^	2.23(0.30)
Collagen 3 SQS ASM^^	0.56(0.1)	0.68(0.08)	0.58(0.14)	0.43(0.11)	0.68(0.13)
Collagen 3 SQS ASM (number positive)	7/18	6/14	5/10	7/15	7/10
Mast cells/mm^2 ASM	3.1(0.63)	1.03(0.41)	8.8(2.3)*^	11.7(2.3)*^	15.6(2.6)¶*^

Data expressed as mean (SEM). SQS= Semi Quantitative Score

RBM= Reticular basement membrane LP= Lamina propria.

¶ $p<0.05$ one-way ANOVA ; Normal vs. EB/GINA1, GINA2-4, Refractory asthma.

*$p<0.05$ vs control (Mann Whitney tests/ t tests for intergroup comparisons)

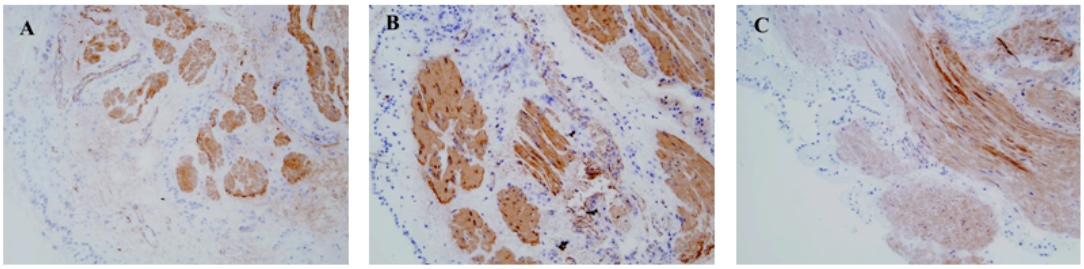
**$p<0.05$ vs GINA 2-4 asthma (Mann Whitney tests/ t tests for intergroup comparisons)

^ $p<0.05$ vs EB (Mann Whitney tests/ t tests for intergroup comparisons)

^^ Mean Score.

Figure 3.5: Increased ASM area in asthma and EB.

*Representative photomicrographs of smooth muscle actin immunostained mucosal biopsies (x100 magnification) in a) Healthy subject, b) EB and c) Refractory Asthma. d) Dot plot of % airway smooth muscle (ASM) in asthmatics with severity defined according to GINA, EB and healthy subjects; *p<0.05.*



d)

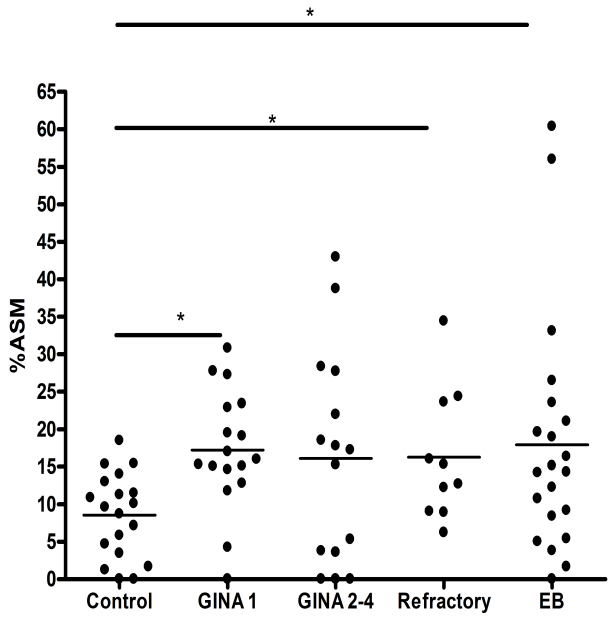
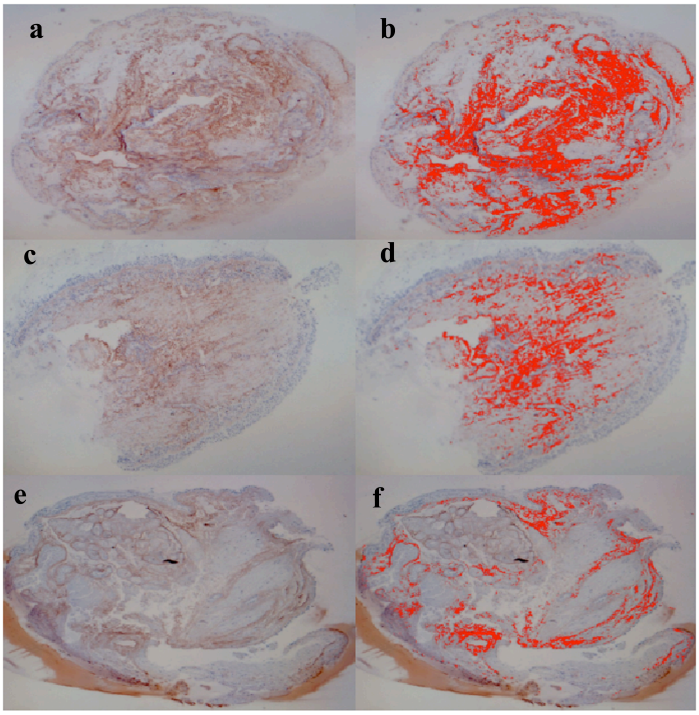


Figure 3.6: Reduced Collagen 3 deposition in the lamina propria in moderate asthma

*Representative photomicrographs of collagen 3 staining (left hand panel) and corresponding thresholded collagen 3 (right hand panel) in the lamina propria in mucosal biopsies in a&b) Healthy subject, c&d) EB, e&f) Asthma (x25 magnification) g). Dot plot of % collagen 3 expression in the lamina propria in asthmatics with severity defined according to GINA, EB and healthy controls; *p<0.05.*



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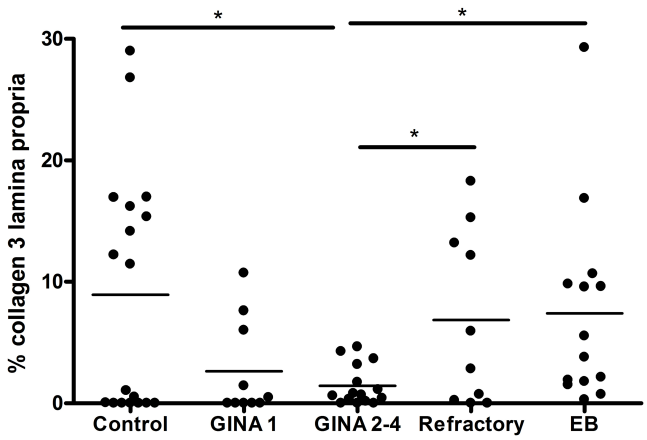


Mast cell localisation to the ASM bundle is present in asthma and with increasing asthma severity.

The mean(SEM) of mast cells/mm^2 of ASM was significantly increased in asthma 11.6[1.4], compared to patients with EB 1.03[0.41] and healthy subjects 3.1[0.63]; p<0.0001 ***Table 3.5 and Figure 3.7a***. The mean(SEM) of mast cells/mm^2 of ASM was significantly increased in asthma independent of severity GINA 1 [8.8(2.3)], GINA 2-4 [11.7(2.3)], refractory asthma [15.6(2.6)] compared to patients with EB and healthy subjects; p<0.0001 (ANOVA) & p<0.05 intergroup comparisons. We did not observe localisation of neutrophils to the ASM bundle in asthma, EB or healthy subjects. Eosinophils were present in the ASM bundle in low numbers in refractory asthma 2.4(0.84)/mm^2 in contrast to GINA1-4 asthmatics, EB and healthy subjects who had no evidence of ASM eosinophilia.

Mast cell localisation to the ASM bundle and not structural remodeling correlates with AHR in asthma.

Univariate analysis demonstrated that mast cell numbers in the ASM was the only feature of remodeling that was associated with log PC_{20} β=-0.51; p=0.004 **(*Table 3.6 and Figure 3.7b*)**. Stepwise linear regression revealed that a combination of mast cell numbers in the ASM [β= -0.43] and disease duration [β= -0.25]; model adjusted R^2=0.26; p=0.027; best modelled log PC_{20}.

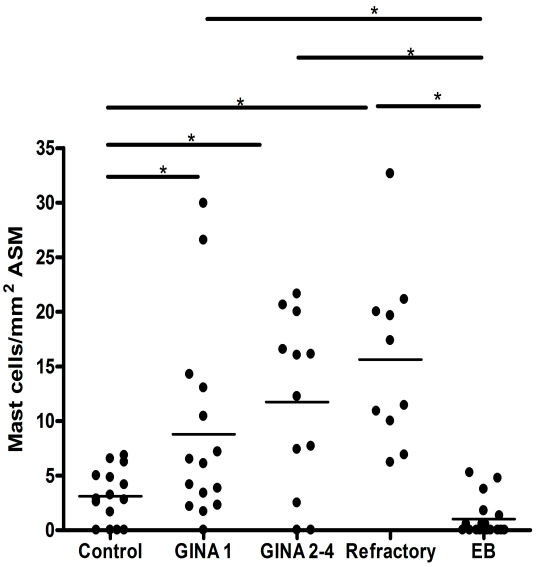
Eosinophilic airway inflammation is associated with thickening of the RBM in asthma and EB.

Univariate analysis revealed that RBM thickening in the pooled asthma and EB cohort correlated with log sputum eosinophil% β=0.29; p=0.03 but not lamina propria eosinophilia β=0.18; p=0.20. Stepwise linear regression revealed that this association was independent of age of onset, disease duration and the BDP equivalent dose of inhaled corticosteroid; β= 0.36, model adjusted R^2=0.1; p=0.04

Figure 3.7: Mast cell numbers in the ASM in asthma correlate with AHR

*a) Dot plot of mast cell numbers [horizontal bar=mean] in the ASM in controls, asthmatics with severity defined according to GINA, refractory asthma and patients with EB;*p<0.05. b) Correlation of mast cell numbers in the ASM (x-axis) and methacholine PC_{20} in asthma (y-axis).*

a)



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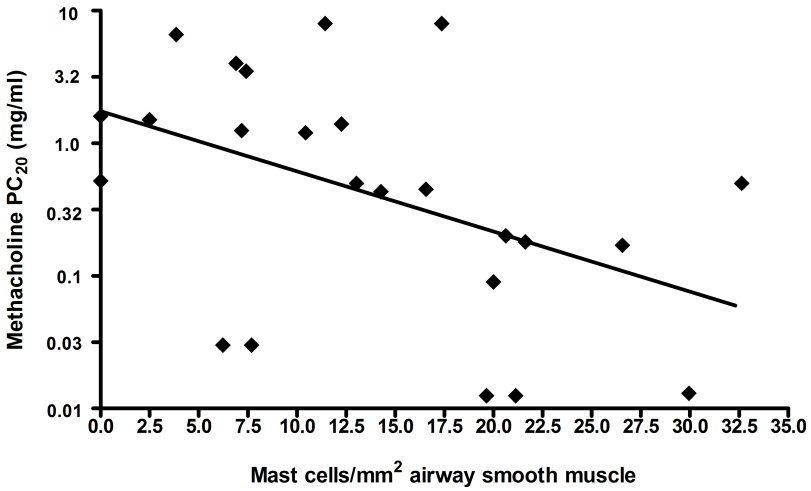


Table 3.6: Univariate and multivariate correlation of airway structure and function in asthma.

	Post Bronchodilator FEV_1 %		**Bronchodilator Response FEV_1 (%)**			**Methacholine PC_{20} (mg/ml)**		
	Univariate analysis		***Univariate analysis***			***Univariate analysis***		
	β	p Value	β	p Value	**β****	β	p Value	**β****
Reticular basement membrane thickness (µm)	0.22	0.18	-0.14	0.40	x	0.16	0.34	x
% Airway Smooth Muscle	0.20	0.22	-0.053	0.74	x	0.16	0.93	x
% Glands Lamina Propria	-0.26	0.10	0.10	0.55	x	0.12	0.50	x
% Collagen 3 Lamina Propria	0.18	0.92	-0.072	0.69	x	0.05	0.81	x
Lamina Propria eosinophils cells/mm^2	-0.09	0.60	**0.32**	**0.048**	**0.31 (p=0.013)**	0.32	0.06	x
Lamina Propria neutrophils cells/mm^2	0.20	0.20	-0.064	0.70	x	0.13	0.45	x
Mast cells/mm^2 Airway Smooth Muscle	-0.03	0.89	-0.024	0.89	x	**-0.51**	**0.004**	**-0.43 (p<0.0001)**

β = standardised correlation coefficient.

β** Stepwise linear regression model adjusted standardised coefficient of correlation

Discussion

We have demonstrated for the first time that structural remodeling of the airway wall, notably increased ASM mass occurs to similar degrees in EB and asthma. In addition we did not observe differences in glandular area or collagen 3 deposition between EB and asthma and confirm that RBM thickening is a feature of both conditions. This suggests that all of these features of the remodeling process in asthma can be dissociated from AHR. In contrast mast cell numbers were increased in the ASM-bundle in asthma only; independent of disease severity and treatment and were shown to correlate with the degree of AHR. Finally eosinophilic inflammation in the airway wall and in induced sputum was related to RBM thickening in asthma and EB.

Our data adds further support to the growing body of evidence that indicates a key pathophysiological role for mast cells in the ASM-bundle in asthma [reviewed in[106]] and strengthens our previously reported association of mast cell infiltration of the ASM-bundle and AHR in asthma[105]. Furthermore in keeping with a recent report[327] we have demonstrated that mast cell localisation to the ASM-bundle was present independent of disease severity and treatment. We observed a non-significant trend for increased mast cells in the ASM-bundle in severe disease. This would suggest that this aspect of the remodeling process may not be modulated by inhaled or oral corticosteroids.

In addition to the relationship between AHR and mast cell localisation to the ASM-bundle, AHR was also associated with disease duration. Indeed the combination of these two factors best modelled AHR. In those subjects with the greatest disease duration, disease onset was often in childhood. It is recognised that AHR may precede the development of asthma symptoms in children and persist into adulthood[376;377]. Thus whether the association between disease duration and AHR reflects increased AHR in early onset disease or the disease burden over time remains to be determined. Importantly, the multivariate regression coefficient between mast cells in the ASM, disease duration and AHR in asthma indicated that a substantial proportion of the variance in the model was due to other factors. AHR is a consequence of complex

interactions between a number of factors and further work is required to define their relative contribution.

We did not find any association between structural remodeling (increased ASM mass, glandular hyperplasia, RBM thickening, collagen 3 deposition and glandular hyperplasia) and AHR in asthma. Furthermore the presence or structural remodeling of these airway compartments to a similar degree in EB strengthens our view that structural remodeling of the proximal airway wall can be dissociated from AHR. In keeping with this observation we have previously demonstrated that vascular remodeling of the proximal airway wall and expression of VEGF[368] are present to similar degrees in asthma and EB again supporting the notion that compartmental remodeling of the airway wall can be dissociated from AHR.

Importantly, bronchial biopsies enable the detailed assessment of the structural components of the proximal airway but cannot determine whether these features of remodeling are associated with changes in the airway geometry. Imaging studies of the airway wall provide global measures of airway geometry. Niimi et al have demonstrated that thickening of the RB1 was inversely associated with airway reactivity (the slope of the methacholine–airway resistance dose response curve), suggesting that global remodeling of the airway wall may be protective against AHR[67]. In contrast Boulet et al have demonstrated that thickening of the airway wall in asthma is associated with increased airway responsiveness in patients with fixed airflow obstruction but not in patients with near normal lung function[378]. The apparent discordance between these imaging studies may lie in the fact that airway reactivity and AHR may be linked to different mechanical properties of the airway wall. Further studies linking measures of static airway geometry by imaging and mucosal biopsies in the same patients are required to further define the association of structure and function.

We did not find an association between RBM thickening, matrix deposition and increased ASM mass with airflow limitation in asthma, in contrast glandular hyperplasia was significantly associated with airflow limitation in asthma. We have

previously demonstrated that vascular remodeling and increased expression of VEGF[368] and a number of groups have demonstrated that global remodeling of the RB1 bronchus is associated with post bronchodilator lung function in asthma[63;68;96]. Importantly our subjects with severe refractory asthma did not have fixed airflow obstruction and this may explain the lack of association between structural remodeling and post bronchodilator lung function reported in other studies[76.]

We have confirmed that eosinophilic airway inflammation is independently associated with thickening of the RBM in both asthma and EB. This would support the growing body of evidence from animal models of asthma[162] and from immunopathological studies of eosinophilic versus non eosinophilic asthma that have linked this feature of the remodeling process to eosinophilic airway inflammation[161].

One potential limitation of the present study is the cross sectional design and the need to confirm our findings in longitudinal studies of airway wall remodeling. However we are confident that our findings are robust. We used non stereological measures of ASM mass and quantified collagen 3 deposition as a proxy for total matrix deposition in the lamina propria and ASM. However the measurements of ASM area correlated well with the ASM volume fraction measured using the stereological Cavalieri method[354]. Furthermore the measurement of collagen 3 using a validated and automated repeatable thresholding procedure correlated well with a previous study that demonstrated similar collagen 3, collagen 1 and total collagen deposition in the lamina propria in asthmatics across the spectrum of severity and healthy subjects, using an automated thresholding procedure[95]. Interestingly, in keeping with our findings this study demonstrated a similar trend for reduced collagen 3 deposition in moderate asthma.

In conclusion we have shown for the first time that remodeling of the airway wall is similar in asthma and EB and dissociated from AHR. In contrast mast cell localisation to the ASM-bundle was associated with AHR in asthma independent of disease severity, treatment and airway wall structure. Further studies are required to establish how global airway wall thickening and compartment remodeling of the airway wall

differentially alter the micromechanical properties of the airway wall in asthma and translate into disordered airway function.

3.1.3

Airway smooth muscle hyperplasia in asthma: a consequence of fibrocyte recruitment?

Abstract

Airway smooth muscle hyperplasia is a hallmark of asthma. It is associated with disease severity and may be related to persistent airflow obstruction. We have considered whether recruitment of fibrocytes, a population of peripheral blood mesenchymal progenitors, contributes to the development of airway smooth muscle hyperplasia. **Method** We assessed the number of fibrocytes bronchial biopsies from subjects with asthma, non-asthmatic eosinophilic bronchitis or healthy controls. **Findings** Twenty-four subjects with asthma and 24 controls were studied. In only severe refractory asthma was the number of fibrocytes increased compared to healthy controls and those with non-asthmatic eosinophilic bronchitis in the lamina propria (1.9[1.7] versus 0[0] and 0[0.5]/mm^2; $p<0.0001$) and airway smooth muscle bundle (3.8[9.4] versus 0[0] and 0[0]/mm^2; $p<0.0001$) respectively. There was a strong correlation between fibrocyte number in the lamina propria and asthma severity defined by the global initiative for asthma treatment step ($r=0.68$; $p=0.0003$). There was no correlation between fibrocytes in the ASM/lamina propria and airway function (PC_{20}/post bronchodilator FEV_1%) **Interpretation** This study provides the first evidence that recruitment of fibrocytes to the airway smooth muscle compartment may be pivotal in the development of the airway smooth muscle hyperplasia that characterises asthma.

Introduction

Asthma is a common disease and remains a significant cause of morbidity and mortality worldwide. It affects 10% of children and 5% of adults, and its prevalence continues to rise[10]. Severe refractory asthma accounts for about 10% of asthma, but is particularly important as it leads to debilitating chronic symptoms despite optimal standard asthma treatment and contributes to over half of the health care costs attributed to asthma[9;11]. There is increasing recognition that asthma is a heterogeneous condition that consists of several domains including clinical expression of the disease, disordered airway physiology and chronic inflammation[5]. One important feature of severe disease is the development of persistent airflow obstruction, which is refractory to current therapy and contributes significantly to the morbidity of the disease.

This persistent airflow obstruction is considered to be a consequence of airway inflammation together with airway remodeling[11]. The airway inflammation in asthma is typically eosinophilic, with increased expression of Th_2 cytokines[100] and mast cell localisation within the airway smooth muscle (ASM) bundle[105]. Airway remodeling encompasses several structural changes in the airway wall including reticular lamina and basement membrane thickening, an increased number of subepithelial myofibroblasts and increased ASM mass. This latter feature is due to a combination of both ASM hyperplasia[88] and hypertrophy, which increases with disease severity[76]. Increased ASM mass is well recognised within both large and small airway walls and is evident in both fatal and non-fatal cases of asthma[32]. It is the most important abnormality responsible for the increased airflow resistance observed in response to bronchoconstricting stimuli[21] and is a major determinant in the development of persistent airflow obstruction in chronic disease[76].

The cause of ASM hyperplasia in asthma is unknown, but is often attributed to increased proliferation. Indeed ASM proliferation is increased in *ex vivo* asthmatic ASM[379], but several reports have been unable to demonstrate increased ASM proliferation *in vivo* [76;88]. An alternative explanation is that ASM progenitors either located within the airway wall or derived from peripheral blood mesenchymal cell

progenitors (fibrocytes)[181], migrate to the ASM-bundle and differentiate into ASM. In support of this view myofibroblasts expressing fibrocyte markers have been identified following ovalbumin challenge in a mouse model of asthma and after allergen challenge in human disease[180].

We hypothesised that in asthma the number of fibrocytes is increased in the ASM-bundle. To test our hypothesis we enumerated the fibrocyte number in bronchial biopsies from subjects with mild-severe refractory asthma, a disease control group; non-asthmatic eosinophilic bronchitis characterised by chronic cough, eosinophilic airway inflammation without evidence of variable airflow obstruction or airway hyperresponsiveness[364] and healthy controls.

Materials and Methods

Subjects

Subjects with asthma (n=24), non-asthmatic eosinophilic bronchitis (n=12) and healthy controls (n=12) were recruited from respiratory clinics, hospital staff and by local advertising. Asthma was defined using the current global initiative for asthma (GINA) guidelines and severity classified based upon the GINA treatment steps[10] and the American Thoracic Society (ATS) criteria for refractory asthma[9]. Non-asthmatic eosinophilic bronchits was defined using the current American College of Chest Physicians guideline[364]. Normal subjects had no history of respiratory disease and normal lung function. All subjects were non-smokers with a past smoking history of less than 10 pack years and were free from exacerbations for at least 6 weeks. The Leicestershire ethics committee approved the study and all patients gave their written informed consent.

Clinical characterisation

Subjects underwent allergen skin prick tests for Dermatophagoides pteronyssinus, dog, cat and grass pollen, spirometry before and after bronchodilator (400µg inhaled salbutamol), exhaled nitric oxide [(eNO) measured at 50mls/s NIOX; Aerocrine, Stockholm, Sweden], methacholine airway responsiveness using the tidal breathing

method (0.03 to 16 mg/ml) followed on recovery by sputum induction using incremental concentrations of nebulised hypertonic saline 3, 4 and 5% each for 5 minutes [241].

Clinical characteristics are shown in table 3.7.

Subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines[352]. Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae; were fixed in acetone and embedded in glycomethacrylate as described previously [353].

Immunohistochemistry to identify fibrocytes

Sequential 2μm sections were cut and stained using monoclonal antibodies (mAb) against CD34 (R&D systems, Oxfordshire, UK), collagen I (Chemicon, Temecula, CA, USA), α-smooth muscle actin and tryptase for mast cells, or appropriate isotype controls (DAKO, Cambridgeshire, UK). The number of positive nucleated cells was enumerated per mm^2 of lamina propria or ASM-bundle by a blinded observer. Fibrocytes were identified as Collagen I^+ $CD34^+$ cells determined by the technique of co-localisation as described previously[142] (see methods section).

Analysis

Statistical analysis was performed using Prism Version 4 (Prism, San Diego, Calif). Parametric data were expressed as mean (SEM), data that had a log normal distribution was log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (range). One-way analysis of variance and Kruskal-Wallis test were used for comparisons across groups with post-hoc Tukey's and Dunn's comparisons between groups for parametric and non-parametric data respectively. Chi squared tests were used to compare categorical data. Pearson and Spearman Rank correlation coefficients were used to assess correlations. A p value of <0.05 was taken as the threshold for statistical significance.

Table 3.7. Clinical characteristics

	Control (n=12)	Eosinophilic Bronchitis (n=12)	Mild to Moderate Asthma (n=12) GINA 1 (6) GINA 2-4 (6)	Refractory Asthma (n=12)
Age(yrs)	36 (17)	49 (13)	33 (24)	49 (6)
Sex M:F	4:8	4:8	6:6	5:7
Atopy (%)	42	50	75	67
Disease Duration (yrs)	N/A	5 (8)	14.5(11.0)	20.2 (15.9)
Smoking (pack years)	0(0)	5 (3)	4.0(5.9)	3.4 (1.8)
Inhaled BDP (mcg/24hrs)	N/A	266 (420)	520 (658)	1633 (458)
Oral Prednisolone^	N/A	0; 0/12	0; 0/12	5.5 (6.6); 7/12
LABA (%)	N/A	0	25	100
FEV_1 (L)	3.46 (1.04)	2.80 (0.64)*	2.87 (1.11)*	2.50 (0.78)*
FEV_1 % predicted	102.3 (9.8)	95.7 (11.1)	89.6 (19.9)*	81.4 (6.3)*
FEV_1/FVC	87.7 (12.0)	81.1 (6.5)	79.9 (11.4)	72.9 (12.0)*
Bronchodilator Response (%)	0.6 (2.2)	-1.0 (2.9)	8.5 (15.9)*	4.6 (14.0)*
$PC_{20}FEV_1$ methacholine (mg/ml) #	>16	>16	0.36 (0.03-2.34)*	0.40 (0.06-2.5)*
FE_{no} (50mls/s) ppb #	16 [9-28]	17 [10-30]	20 [12-25]	29 [16-53]
Sputum eosinophils (%)^	0.25 (0.75)	6.2 (11.8)*	0.25 (4.1)	5.9 (39)*
Sputum neutrophils (%)^	49.3(37.9)	50.1 (37.9)	52.4(27.5)	50.1 (43.1)
Mast cells/mm^2 ASM ^	3.8 (4.6)	0 (0.3)	9.8 (17.8)	11.4 (14.2)

*Data expressed as Mean (SD) except for #Geometric Mean [95% Confidence Interval] and ^median (IQR). BDP equivalents (Beclamethasone Diproprionate/24 hrs); fluticasone 2:1, Budesonide 1.25:1, Mometasone 1.25:1. *ANOVA or Kruskal-Wallis as appropriate with post-hoc tests for multiple comparisons. LABA= Long acting bronchodilator. ^^ mg/24 hrs; number taking oral corticosteroids.*

Results

Fibrocyte numbers are increased in the ASM-bundle and lamina propria in asthma

Representative photomicrographs of $CD34^+$Collagen $I^+\alpha$-smooth muscle $actin^+$ fibrocytes in the ASM-bundle and lamina propria are as shown (Figure 3.8). The ASM-bundle area was insufficient to assess fibrocyte number in 2/12 subjects with non-asthmatic eosinophilic bronchitis and 1/12 healthy controls. The median (IQR) number of fibrocytes/mm^2 in the lamina propria was increased in those with refractory disease (1·9 [1·7]) compared to mild-moderate asthmatics (0 [0.4]), non-asthmatic eosinophilic bronchitis (0 [0.5]) and healthy controls (0 [0]) ($p<0.0001$; figure 3.9a). The % ASM area was increased in those with refractory 19.5 (3.6)% and mild-moderate asthma 23.3 (1.9)% compared to healthy controls 10.8 (1.5)%, but was not significantly different in non-asthmatic eosinophilic bronchitis 14.6 (2.5)% ($p=0.004$ ANOVA; $p<0.05$ both asthma groups versus healthy controls). The number of fibrocytes/mm^2 was markedly increased in the ASM-bundle in subjects with severe refractory asthma (3.8 [9.4]) compared to mild-moderate asthma (1.1 [2.4]), subjects with non-eosinophilic bronchitis (0 [0]) and healthy controls (0 [0]) ($p<0.0001$; Figure 3.9b). The number of fibrocytes and mast cells within the ASM-bundle was correlated for the whole group ($r=0.7$; $p<0.0001$), but not in asthmatics alone. The fibrocyte number in tissue did not correlate with spirometry, airway hyperresponsiveness, sputum eosinophil count or ASM mass (Table 3.8).

Figure 3.9: Fibrocytes are located in the lamina propria and ASM-bundle in asthma.

Representative photomicrographs of bronchial biopsies illustrating a) negative isotype control (x200), and (b-f) fibrocytes in bronchial biopsies from 2 different asthmatics identified in the lamina propria by sequential sections stained for b) CD34, and c) collagen I and in the ASM-bundle stained for d) α-smooth muscle actin, e) CD34, and f) collagen I (x400). Fibrocytes highlighted by arrows.

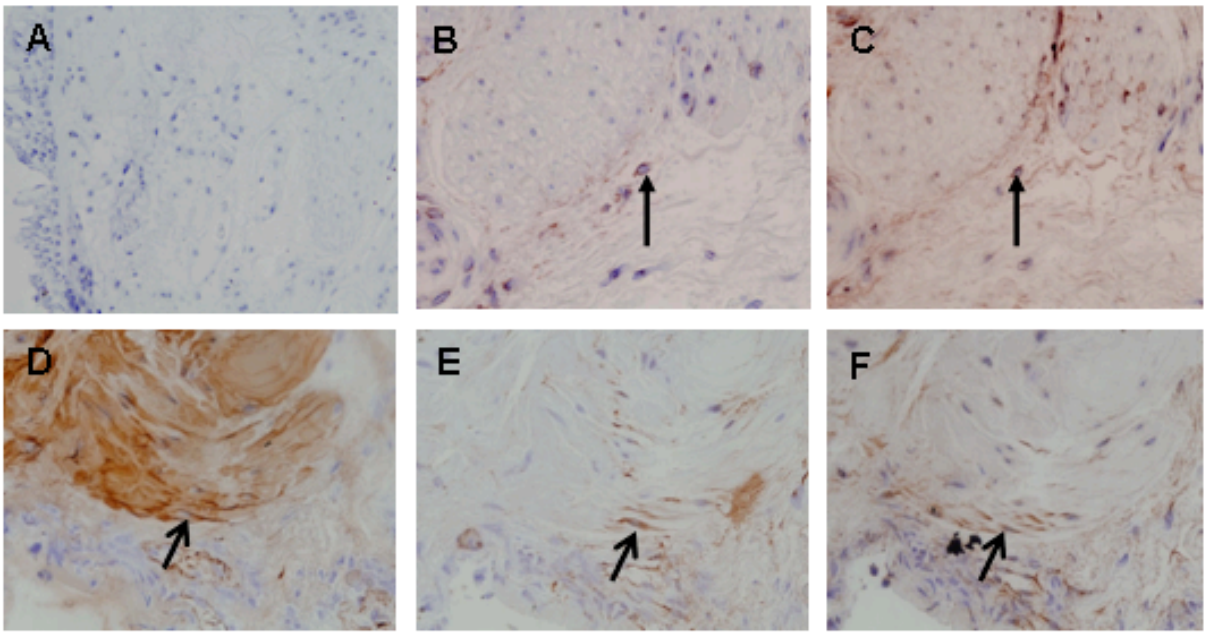


Figure 3.10: Dot-plot of the number of fibrocytes in a) the lamina propria and b) ASM-bundle in bronchial biopsies from subjects with asthma and healthy controls.

Horizontal Bars-Median. p<0.05 Kruskal-Wallis test; ^p<0.05 Dunns post test.

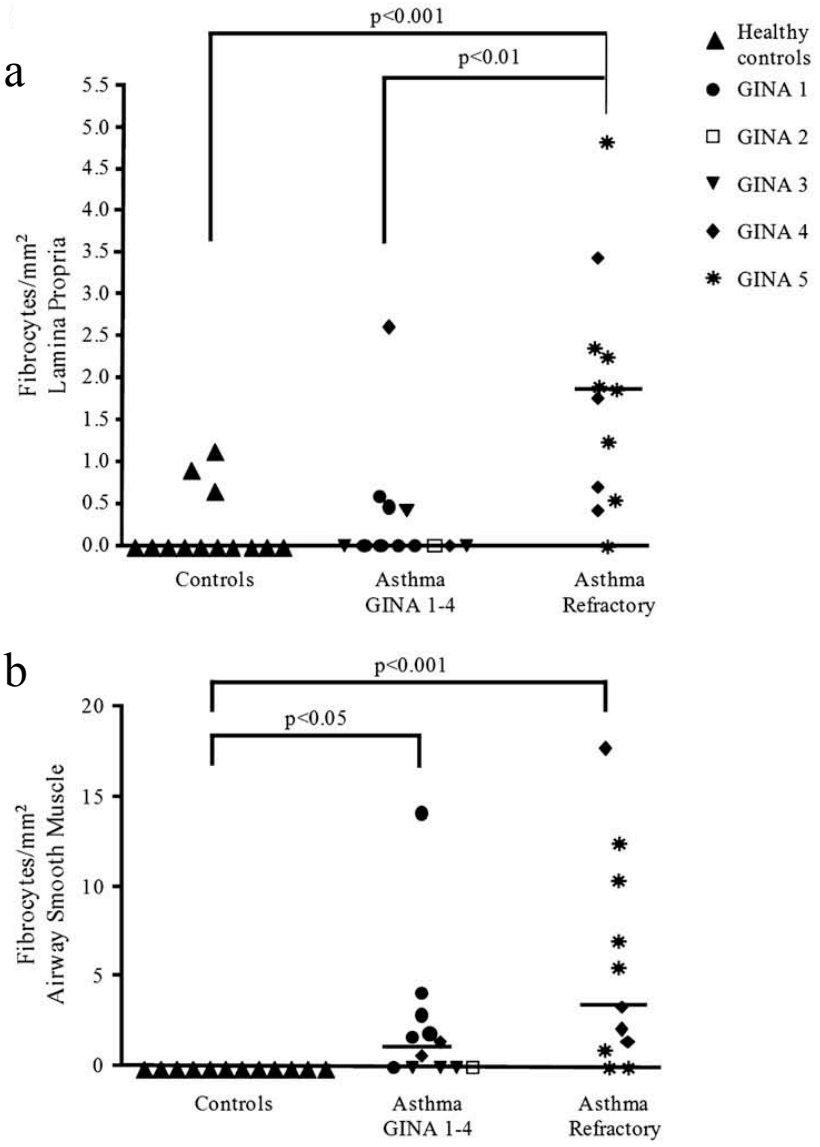


Table 3.8: Relationship between tissue fibrocytes and airway function in asthma.

Spearmans Correlation (r_s)	**Post bronchodilator FEV_1 % predicted**	**Methacholine PC_{20} (mg/ml)**
Fibrocytes/ mm^2 lamina propria (all asthmatics)	-0.28; p=0.20	0.16; p=0.49
Fibrocytes/ mm^2 lamina propria (n=5 asthmatics with fixed airflow obstruction)	-0.80; p=0.13	-0.2; p=0.78
Fibrocytes /mm^2 ASM (all asthmatics)	-0.08; p=0.71	0.30; p=0.20
Fibrocytes /mm^2 ASM (n=5 asthmatics with fixed airflow obstruction)	0.40; p=0.52	0.87; p=0.08

Discussion

In this study we report for the first time that in severe refractory asthma the number of fibrocytes is increased in the bronchial lamina propria and in the ASM-bundle. This is the first study to provide evidence that recruitment of ASM-progenitors to the ASM compartment may be pivotal in the development of the increased ASM mass that characterises asthma.

To date ASM hyperplasia in asthma has been largely attributed to increased ASM proliferation. Although this view finds support from primary culture of ASM from asthmatics[355;379] there is currently no evidence in bronchial biopsies of increased ASM proliferation[76;88]. Possible explanations for this lack of evidence are that our current tools are too blunt to identify small changes in ASM proliferation or that the proliferation only occurs in response to specific stimuli, perhaps at exacerbations, limiting our opportunities to observe it. We propose an alternative explanation that ASM hyperplasia may be a consequence of the recruitment of progenitors to the ASM-bundle. Our findings support this view as the number of fibrocytes was increased in the ASM-bundle in asthma compared to healthy controls and subjects with non-asthmatic eosinophilic bronchitis with this increase most marked in severe refractory disease. Interestingly we report here a non-significant increase in ASM mass in non-asthmatic eosinophilic bronchitis, which is in keeping with earlier work suggesting that increased ASM mass is a characteristic of this condition[380]. The paucity of fibrocytes in the airway wall in non-asthmatic eosinophilic bronchitis suggests that fibrocyte recruitment to the ASM-bundle is a feature of asthma rather than the presence of airway inflammation per se. This extends the earlier observation that in mild disease the number of lamina propria fibrocytes increased following allergen challenge[180].

The view that ASM hyperplasia may be a consequence of fibrocytes trafficking to the airway is consistent with mechanisms proposed in skin wound healing[181;381] and is analogous to the current concepts of cardiac myocyte progenitors contributing to cardiac repair following myocardial infarction[382]. In addition to their role in tissue repair fibrocytes have been implicated in lung fibrosis[383]and other fibrotic diseases (reviewed in[384]). In mouse models of lung fibrosis fibrocytes, are the

major source of matrix protein deposition[385] and blockade of fibrocyte recruitment inhibits the development of lung fibrosis[383]. Fibrocytes in tissue differentiate into myofibrocytes and express α-smooth muscle actin. In asthma, they therefore have the potential to contribute to the pool of lamina propria myofibroblasts and have the capacity to migrate to different airway compartments. Whether fibrocytes in the ASM-bundle remain as immature progenitors or mature fully into ASM has yet to be determined.

The relationship between airway structure and function is contentious leading to considerable debate over the clinical importance of airway remodeling[386]. Indeed we have recently reported that airway remodeling including ASM-mass was not associated with airway hyperreponsiveness[380]. However, airway wall thickening, assessed by CT imaging, is strongly and consistently correlated with airflow obstruction in asthma and chronic obstructive pulmonary disease (COPD)[69;96]. Importantly, mathematical models suggest that thickening of the airway wall is fundamental in the development of disordered airway physiology[290]. Features of airway remodeling in asthma notably increased ASM mass[76], increased vascularity[368], thickening of the reticular laminar and basement membrane and fibroblast numbers in the lamina propria[76], have all been shown to correlate with airflow limitation. In most studies these features are studied in isolation limiting our ability to determine the relative contribution of each component of remodeling with lung function. This shortcoming has perhaps been best overcome by Benayoun et al[76] who carefully evaluated several features of airway remodeling and found that subepithelial fibroblast number and increased ASM mass were the major determinants of persistent airflow obstruction. Critically, this earlier report did not distinguish between resident mesenchymal cells and recruited fibrocytes. More recently Wang et al have demonstrated that there are increased numbers of circulating fibrocytes in asthmatics with airflow obstruction compared to asthmatics without airflow limitation or healthy controls. In this study the increase in fibrocyte numbers correlated with FEV_1 decline. Furthermore the release of TGF-β was higher in fibrocytes from asthmatics with airflow obstruction and anti TGF-β inhibited myofibroblastic differentiation of fibrocytes[186]. Interestingly in the small number of asthmatics that had airflow obstruction we found that increased fibrocytes in the lamina propria showed a

non significant trend for increased airflow limitation, in contrast increased fibrocyte numbers in the ASM in this subgroup correlated inversely with the degree of AHR.

One potential limitation of the present study is its cross-sectional design. Airway remodeling occurs dynamically overtime and therefore our findings need to be confirmed in longitudinal studies. This is particularly pertinent with respect to studying the relationship between airway structure and function. For example, we were unable to demonstrate a relationship between fibrocyte infiltration, ASM-mass and measures of lung function. Future studies will need to examine the relationship between fibrocyte recruitment and longitudinal outcomes such as lung function decline. Similarly, although we found striking differences in fibrocyte numbers between asthma and health our study design does not enable us to determine whether the differences in severe asthma reflect severity of disease or treatment. Hence later studies will need to study the effect of therapy within individuals. In spite of these shortcomings we are confident that our findings are robust as the fibrocyte cell counts in tissue were enumerated by observers blinded to the clinical characteristics. Finally we did not determine the mechanisms of recruitment of fibrocytes to the ASM bundle in asthma and future work will be necessary to establish how fibrocytes home to the ASM in asthma and the mechanisms of increased ASM mass seen in EB.

In summary, we report for the first time that in severe asthma the number of fibrocytes is increased in the bronchial lamina propria and in the ASM-bundle. This is the first study to provide evidence that recruitment of ASM-progenitors to the ASM compartment may be important in the development of increased ASM mass that characterises asthma and therefore presents a novel target in the treatment of asthma.

3.2 Airway Wall Geometry in Asthma and EB.

3.2.1 Validation of multidetector computed tomography to measure cross sectional geometry of the RB1 bronchus: A phantom study.

Abstract

Limited computed tomography of the RB1 bronchus (apical segmental bronchus right upper lobe) may have utility as an indirect measure of airway wall remodeling in asthma. We designed a phantom airway modelling RB1 and cross sectional geometry of airways (generations 3-12) in asthma and health. We sought to assess the accuracy and repeatability of manual and automated measures of cross sectional airway wall geometry and attempted to derive ways of predicting and minimising observer error. **Methods.** A phantom model of 9 plastic tubes of varying airway wall dimensions [Range wall area (WA) ±2SD normal and asthmatic airways (2.42-47mm^2)] was constructed. Gold standard measurements were made using a combination of stereomicroscopy; Vernier callipers and Micro-CT. Cross-sectional measurements were made at a face designated the leading face using a manual algorithm and with an automated airway analysis program using the full-width at half-maximum (FWHM) technique by 3-blinded observers. **Results.** Manual and automated cross sectional measurements were highly repeatable and were subject to size dependant errors that could be predicted and corrected. Prediction equations for cross sectional area using FWHM significantly reduced error in a separate *ex vivo* sheep airway model. Corrected cross sectional measurements could be made within 5% absolute error down to a total area of 6.61mm^2 and radius of 1.45mm. **Conclusions.** Airway wall cross sectional dimensions modelling the RB1 bronchus can be measured accurately and repeatably using MDCT.

Introduction

Structural changes within the airway wall known as 'remodeling' may occur in a number of common airway diseases and lead to fixed airflow obstruction and FEV_1 decline[76;350;387]. Multidetector computed tomography (MDCT) has been advocated as an indirect measure of airway wall thickening in a variety of airway diseases[65;69;388-390]. Standard thoracic HRCT is limited by significant size dependant errors primarily but not exclusively due to partial volume averaging. Furthermore the radiation effective dose, questions the validity of serial CTs to assess remodeling longitudinally[391]. Limited computed tomography (CT) of the apical segmental bronchus (right upper lobe) [RB1] offers promise as a low radiation procedure and has previously been used as a technique to measure airway wall thickening in asthma and COPD[67;68;96;392]. Furthermore thickening of RB1 has been shown to correlate with distal airway wall thickening and inversely with airflow obstruction[67-69;96;392].

However the value of limited CT in the measurement of airway remodeling has not been formally validated and strategies to minimise the error related to airway size have not been investigated.

A variety of studies have attempted to validate the measurement of airway wall geometry using *ex vivo* inflated lung tissue[393;394]; however the majority of these demonstrate large absolute errors in geometry measurements compared to gold standards, which are likely to be secondary to tissue shrinkage *ex vivo*. Furthermore the morphometry of the human bronchial tree, particularly with reference to the upper lobes has been shown to be considerably different to that of other mammalian species used to validate airway geometry in *ex vivo* models[395].

We designed a phantom airway model to validate the measurement of airway wall geometry of the RB1 bronchus as well as other airways (generations 3-6) by MDCT. Our aims were to 1) Establish the repeatability and accuracy of airway wall cross sectional dimensions using MDCT, 2) design strategies aimed at minimising observer error, 3) to establish the limit of detection of repeat CT examinations and 4) to quantify the effective radiation exposure of our limited CT scanning protocol compared to commonly performed standard thoracic CT procedures.

Materials and Methods

Cross sectional validation of airway geometry measures

We designed a phantom airway modelling RB1 down to the 12th generation and further sought to validate the phantom model with a separate *ex vivo* sheep airway model.

Airway phantom

The airway phantom (figures 3.10a) was constructed from a polystyrene block with 9 circular plastic tubes, dimensions [Luminal area (LA)(0.95-19.17mm^2); Wall area (WA) (2.42-47.02mm^2); Total area (TA) (3.37-66.19mm^2)] covering the range for WA and LA of RB1, in health and in asthma and of airways generations 3-12[396,68] (tables 3.9&3.10).The polystyrene had a mean attenuation ±(SEM) of –965 HU (9.3), similar to the density of inflated lung tissue. The mean (range) tube wall attenuation was 727 HU (154-1029) (figure 3.11a&b).

Steromicroscopy: Cross sectional gold standard.

An Olympus SZX12 stereomicroscope was used to image and measure the tube geometry at a marked face designated the leading face (figure 3.12a). Measurements were made using Aquis Pro software (Syncroscopy, Cambridge, United Kingdom). Calibration was performed using a standard calibration block, which gave an accuracy of ±1 m. The length of the phantom tubes was measured with Vernier calipers and the mean (range) length was 51.91 (31.48-60.32) mm.

Figure 3.10: Phantom airway model

3.11a: Polystyrene phantom with 9 embedded plastic tubes modelling the RB1 bronchus and smaller airways to the 12th generation.
3.11b: Corresponding CT image of polystyrene phantom with tubes at their leading face.

a) ***b)***

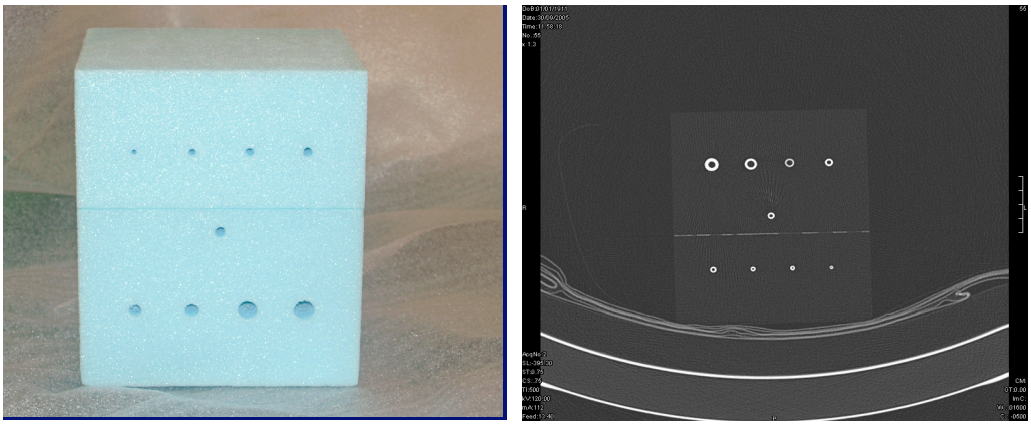
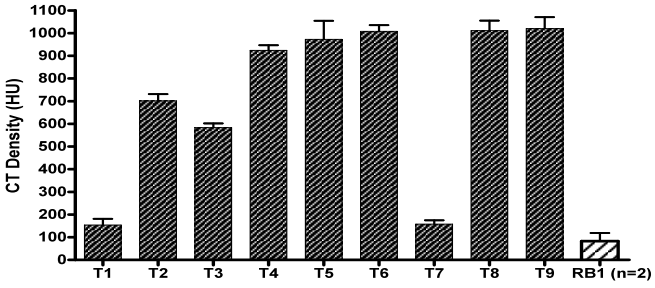
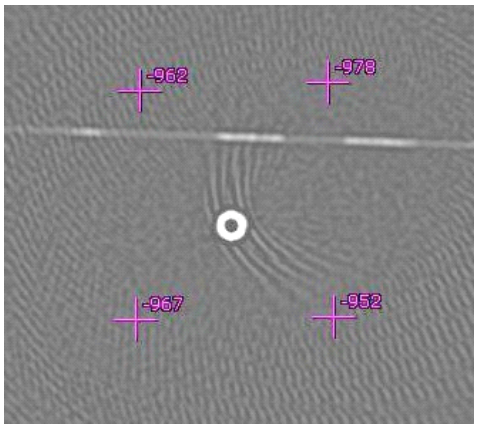


Figure 3.11: CT density of phantom tubes (a) compared to the RB1 bronchus (n=2 healthy subjects) and polystyrene CT density (b).

a) b)

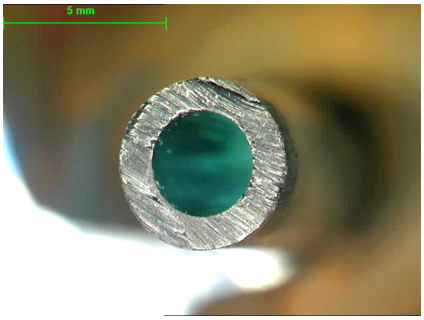




HU=Hounsfield Units

Figure 3.12: Stereomicroscope image of leading face of tube 6 and corresponding CT image (Window Level -450 HU, Window Width 1600 HU)

a) **b)**



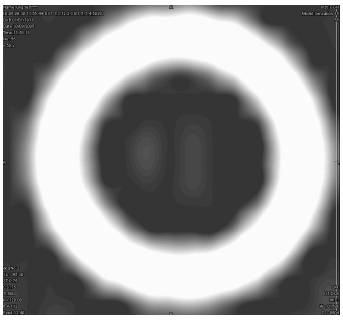


Table 3.9: Expected geometry of RB1 based upon *Niimi et al* AJRCCM 2000

Apical Segmental Bronchus	Expected Range
%WA	41.7-84.4
WA	9.0-47.7
LA	3.0-32.2

Healthy control (Mean -2SD) - Severe asthma (Mean + 2SD)

Table 3.10: Phantom Tube Stereomicroscope leading face geometry

Tube	%WA	WA (mm^2)	LA (mm^2)	WT (mm)
1	71.8	2.42	0.95	0.47
2	64	4.23	2.38	0.58
3	60.68	4.29	2.78	0.56
4	59.67	7.5	5.07	0.73
5	58.35	8.63	6.16	0.77
6	63.29	13.86	8.04	1.04
7	58.39	17.29	12.57	1.09
8	61.33	32.39	20.42	1.55
9	71	47.02	19.17	2.12

WT= wall thickness

WA= Wall area

LA= luminal area

%WA= percentage wall area

Basic CT Physics

The CT image is composed of a matrix (usually 512x512) of pixels. Each pixel is displayed according to the mean x-ray attenuation of the tissue(s) that it corresponds to on a scale from -1024 to +3071 (the Hounsfield scale). When the CT slice thickness is also factored in, the unit is known as a voxel, which is a three dimensional unit. The Hounsfield unit (HU) scale is a linear transformation of the original linear attenuation coefficient measurement in which the radiodensity of distilled water at standard pressure and temperature (STP) is defined as zero HU, while the radiodensity of air at STP is defined as -1000 HU. The HU scale extends from -1024 (air) to +3071 (high density structures eg; metallic implants). The HU of other common tissues is -120 (fat), +40 (muscle) and >300 (bone). Windowing is the process of using the calculated HU to make an image. A typical display device can only resolve 256 shades of grey, some specialty medical displays can resolve up to 1024 greys. These shades of grey can be distributed over a wide range of HU values to get an overview of structures that attenuate the beam to widely varying degrees. Alternatively, these shades of grey can be distributed over a narrow range of HU values (called a *"narrow window"*) centered over the average HU value of a particular structure to be evaluated. In this way, subtle variations in the internal makeup of a structure can be discerned. This is a commonly used image processing technique known as contrast compression. The window width is the range of HU values used to define the image and the median value across the width range is the window level. For the analysis of airways a window level of -450HU has traditionally been adopted with a width of 1200-1600HU[68]. Therefore for a window level of -450HU and width of 1200 HU any pixel/voxel with a HU below -1024HU would be displayed as pure black and any pixel/voxel with a HU greater than +150 would be displayed as pure white.

CT scanning

CT scanning was performed with a Siemens Sensation 16 MDCT scanner. Scans were obtained at both 16x0.75mm collimation, 120 kV, 50 mAs with a table feed of 13.5mm per 0.5 second scanner rotation. Images were reconstructed at 0.75 mm spacings using a 139 mm field of view with a 512 x 512 matrix and were visualised at settings appropriate for lung parenchyma (window level of –450 HU and window width of 1600 HU) using a B70-f very sharp algorithm (figure 3.10b&3.12b). The voxels size was 0.27x0.27x0.75mm.

Manual cross sectional validation and windowing analysis

Images were exported to Image J version 1.32 (NIH, USA) and magnified prior to measurement, blinding observers to tube size.

Windowing analysis to examine manual error

Optimal window level (WL) and widths (WW) giving the smallest % error in tube areas were detemined by a single blinded investigator in a detailed windowing examination. Mean % errors in WA measurements were derived for each tube in the following WW/WL combinations; 1) WL fixed at –450HU, WW 0-1600HU, 2) WW fixed at 1600HU, WL –1000- 0 HU[68;397].

Manual validation

3-blinded observers subsequently measured tube dimensions at the leading face (collimation 0.75mm, slice thickness 2mm) at the optimal window level and window width. A mean of 3 measurements/observer were made for: total area and luminal area (best fitting circles), inner and outer diameters and wall thickness radially at 3, 6, 9, 12 pm (the mean of these measurement was used to derive the mean wall thickness).

Automated cross sectional validation

An automated program Emphylyx-J V 1.00.01[398] using the full width at half maximum technique (FWHM) was used to determine the accuracy and repeatability of a non biased objective measure of edge detection on airway wall cross sectional geometry (figure 3.13a). The advantage of using the Emphylyx computer algorithm is that it is automated and uses all the Hounsfield units/all grey levels in the CT datset and is therefore completely independent of windowing width and level. 3 observers measured tube dimensions using the FWHM technique. A central seed point was placed in the tube lumen; 64-128 rays cast out radially from this seed point were used to delineate the inner and outer wall according to the full width half maximum method (figure 3.13 b-d). Image data were transferred from the CT workstation to a personal computer in DICOM 3.0 format. Briefly the program implemented the following steps:

1) Sorting the DICOM files: The header portions of the DICOM files are read and the files sorted according to slice number.

2) Displaying the CT image: The user chooses one slice from the sorted list of files and the corresponding CT image is displayed on the screen. The gray scale of the image can be adjusted if necessary.

3) Enlarging the image: The user selects the center of a region of interest and this region is enlarged and displayed.

4) Selecting the airway: The user indicates an airway for measurement by clicking the mouse inside its lumen.

5) Airway wall recognition: The clicked point inside the lumen is labeled as a "seed point". A ray is cast outward from this seed point to the airway wall and beyond into the lung parenchyma. The CT numbers along the ray are examined to determine the inner and outer boundaries of the airway wall using the "full-width at half maximum" (FWHM) principle. The profile of the CT numbers along this ray has a local minimum (G1) in the lumen, a maximum (G Max) in the wall, and a local minimum (G2) in the parenchyma. The middle value ("half- maximum") between the G1 and GMax is calculated and considered as the inner boundary. Similarly, the middle value between GMax and G2 is calculated and considered as the outer boundary. Linear interpolation is used to estimate the boundaries to within one-tenth of a pixel. The distance between

the inner and outer boundaries is defined as the thickness of airway wall along this ray. This process is repeated along 128 (or 64; depending on the size of the airway) different ray directions giving 128 (or 64) values of thickness.
6) Exclusion of shorter and longer thickness: Since airways sometimes run parallel to pulmonary blood vessels whose CT numbers are too high to be separated from the airway wall, the wall thickness cannot be adequately measured in this region of apposition. Therefore, among the 128 (or 64) thicknesses, those longer than an interactively-specified threshold are excluded if necessary. Similarly, thicknesses shorter than a given threshold can be eliminated. Median filtering can also be used to eliminate short thicknesses.
7) Defining airway wall borders: After the exclusion of longer and shorter thicknesses, the inner and outer borders of the airway wall are estimated by splining the endpoints of the remaining thicknesses.
8) Calculation of the airway wall dimensions: The area of the airway lumen (LA) is defined as the area inside the inner border line. The area of the airway wall (WA) is defined as the area between the inner and outer borders. lumen long axis and lumen short axis are also calculated.

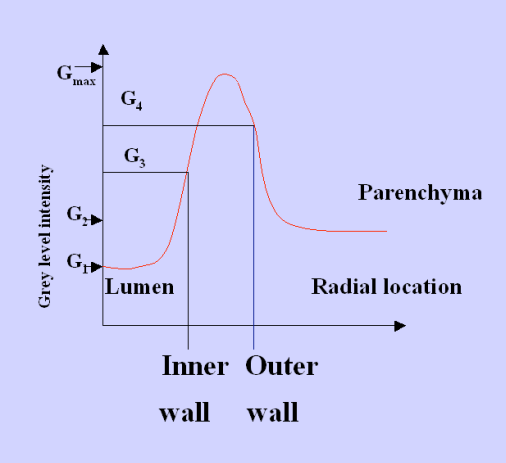
The influence of slice thickness and collimation was assessed by measuring the tubes at 3 combinations of collimation and slice thickness (0.75, 0.75mm; 0.75, 2mm; 2, 1.5mm) to assess the impact of collimation and slice thickness on the accuracy of tubes measurements. The influence of reconstruction algorithm upon tube WA and LA measurements was also assessed by reconstructing phantom tubes at three high spatial frequency algorithms (B70-F, B80-F and B95-UF).

The influence of oblique orientation on wall area measurements was assessed by reconstructing each tube at the leading face at oblique orientations ranging from 0° (strictly perpendicular to the long axis of the tube) to 60° at 10° angular increments, and corresponding to a ratio of maximum: minimum luminal diameter from 1-2 (figure 3.14a-d).

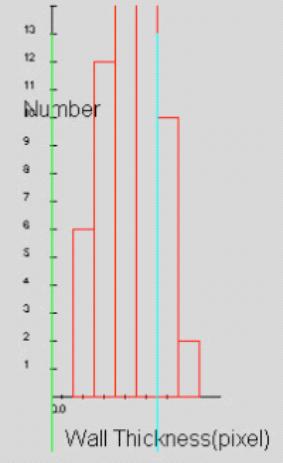
Figure 3.13: Automated edge detection using the Full Width at Half Maximum (FEHM) Method.

128 Rays are cast out radially from a central seed point in the lumen and delineate the inner and outer wall according to the midpoint of grey level transition across the wall edge (a). **The inner wall G3=[(Gmax-G1)/2] & the outer wall G4=[(Gmax-G2)/2]**. *A histogram of wall thickness distribution of cast rays is derived and the entire histogram accepted as the wall measurement. The histogram can be manipulated to exclude rays that are clearly within an adjacent vessel where the CT numbers of the airway and vessel can not be differentiated. (b). The lumen and wall are therefore delineated according to the full width half maximum principle (c). An example of the same technique used to delineate the RB1 bronchus (d).*

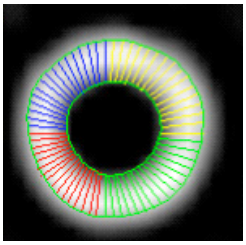
a)



b)



c)



d)

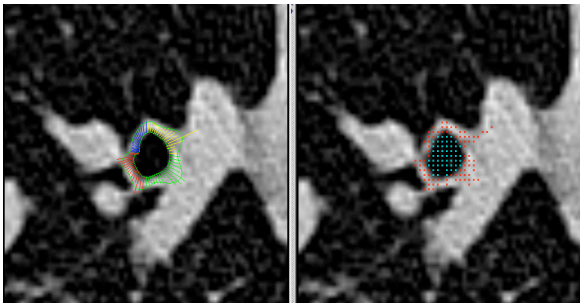
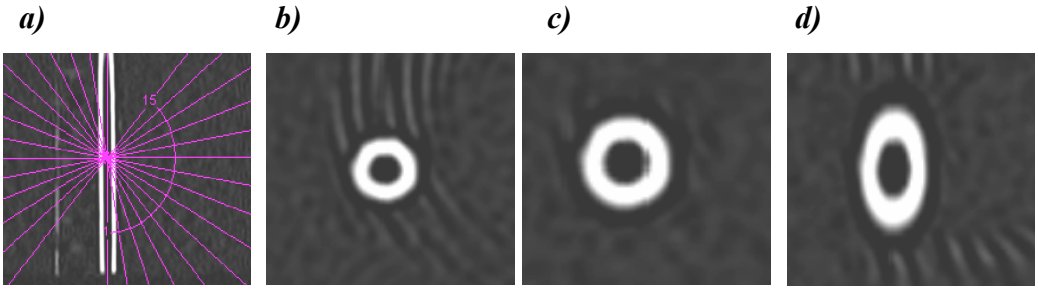


Figure 3.14: Oblique reconstruction of phantom tubes

Example of a phantom tube in the long axis view; reconstructed at incident oblique planes ranging from 0-60° along the central axis of the tube lumen denoted by the confluence of the planes 3.15a. Increasing oblique orientation from 0° (b), 30° (c) & 60° (d) leads to a progressive overestimate of luminal and wall area.



Correction of size dependant error and oblique orientation for geometric measurements of the RB1 bronchus.

For Limited CT scans of the RB1 bronchus we performed a comparison of the total area (TA) and Wall area (WA) obtained by stereomicroscopy for the phantom tubes and the FWHM/manual algorithm to see whether we could derive correction equations to correct for the size dependant error in geometry that is known to occur with the FWHM method as airway size decreases. The corrected luminal area and %WA were derived from the corrected WA&TA; LA=corrected TA- Corrected WA and %WA=corrected WA/corrected TA *100.

For HRCT images of the RB1 Bronchus due to the non-volumetric acquisition and the likelihood of only capturing the RB1 bronchus once across its entire length; we derived a method of correcting both size dependant errors in airway geometry and oblique orientation. We derived correction equations by looking at the best parabolic planar 3 dimensional fit of the 3D plot of phantom tube true wall area/luminal area, the maximum/minimum diameter of the airway lumen [a marker of oblique orientation] and the true wall area/luminal area measured by stereomicroscopy to the nearest micron. For each tube 7 values of maximum/minimum ratio and corresponding geometry (wall area and luminal area) were measured using the full width half maximum (FWHM) method were derived based upon reconstructing each phantom tube from 0º (perpendicular to the long axis of the tube) to 60º corresponding to a ratio of largest to smallest diameter of 1.0 to 2.0. The final 3D equation was derived for all 63 measurements of the 9 phantom tubes reconstructed from 0º (perpendicular to the long axis of the tube) to 60º, by fitting the best parabolic plane to the 3D dataset. 3D datasets and equations were generated using a custom program LeoStatistic Version 14.5 (LeoKrut, Arlignton, USA).

The corrected Total area and % Wall area were derived from the corrected LA/WA.
Corrected TA=True LA+ True WA
Corrected %WA= True WA/Corrected TA

Sheep phantom

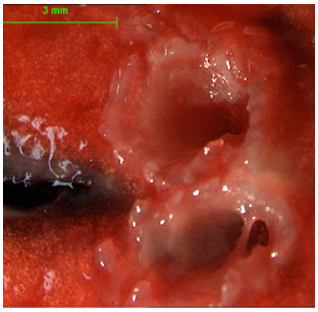
A macroscopically healthy sheep lung was removed from a freshly sacrificed sheep in a local slaughterhouse. The trachea and upper airway were removed and the right and left lungs were cut into 13 slabs of thickness ranging 2-4cm. The slabs were snap frozen at -80^{o}C for 4 hours. The frozen slabs were then imaged using the above CT protocol parallel to their cut face. After scanning 29 airways; Total area (TA) median (range) [14.0(4.1-31.9) mm^2], LA median (range) [3.5(0.86-15.5) mm^2] and %WA median (range) [69.9(44.1-89.7) %] were identified on the leading face of the slabs using stereomicroscopy that could also be easily registered with the CT image of the corresponding airway at the leading face. The CT imaged airways were measured using the FWHM algorithm. (Figures 3.15 a-d).

The sheep airway model was used as a test model to see if error equations generated from the phantom model could successfully reduce cross-sectional error in a separate tissue specific airway model.

Figure 3.15: Sheep Phantom airway Model

3.16a) Stereomicroscope image of 2 sheep airways on the surface of a cut sheep lung slab. b) Corresponding CT image of the surface of the sheep airways on the surface of the cut slab. c) FWHM of the sheep airways using Emphylyxj V1.00.010. d) Frequency distribution of wall and total area of airways in the sheep lung model.

a)



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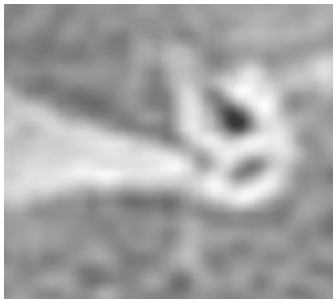

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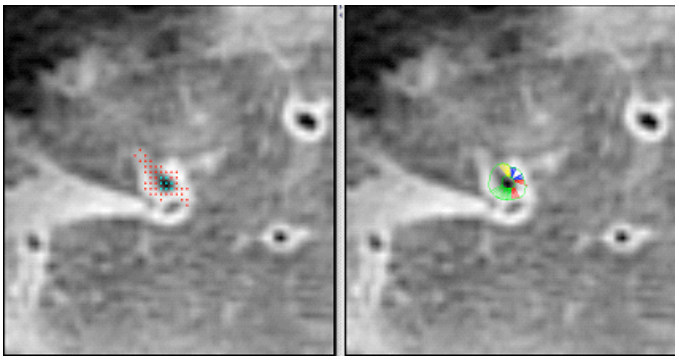
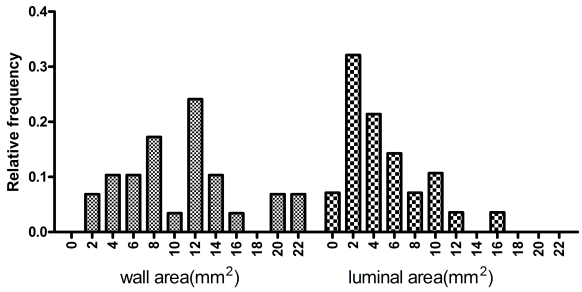


d)



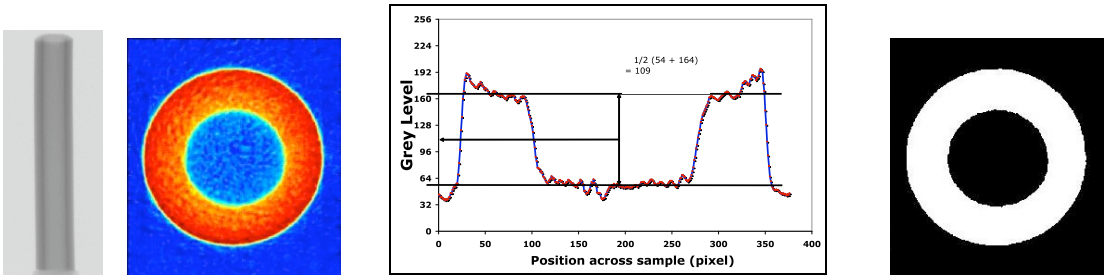
Confirmation of Gold standard geometry using Micro CT.

Micro CT was used to confirm the gold standard stereomicroscope measurements for tube cross sectional areas in the phantom airway model, due to the inherent variability in wall thickness and cross sectional dimensions across the tubes length. The Micro CT analysis allowed us to derive the wall volume and length of phantom tubes. The wall volume/length was used to give a mean cross sectional wall area of the phantom tube.. Measurements were carried out using a high-resolution CT and digital radiography system (HMXST 225, X-Tek Systems Ltd. Tring, U.K) employing a microfocus X-ray source (5μm focal spot size) capable of tube potentials up to 225kV. The imaging arrangement in this system was based on the cone beam geometry and therefore, the voxel resolution of the reconstructed 3-D volume depends on the source-to-object distance. The sample was placed on an object manipulator situated between the X-ray source and the detector system (consisting of image intensifier and CCD), providing magnification and rotation for collection of radiographs over 180 . For the purpose of the material investigated in this study, owing to a relatively low X-ray absorption, a tube potential of 50kV was used with a copper anode target. This, in conjunction with the use of a beryllium windowed detector, ensured attenuation of the X-rays through the polymeric material. In order to shape the energy spectrum of the X-ray source, and thus improve the image quality in the reconstructed slices by increasing the signal-to-noise ratio, a 0.5mm thick aluminium filter was placed in front of the sample during image acquisition. The effect of such an X-ray filter is to remove low-energy photons from the polychromatic beam, thus minimising artefacts in the reconstructed slices and the distribution of voxels with different intensities. Filtration of the beam prior to attenuation through the object thus gives a more uniform distribution of voxel intensities and reduces noise. The 3-D tomographic volumes were reconstructed using a cone beam extension of the filtered back projection algorithm for fan beams from 370 radiographs acquired using a sample rotation step of 0.5 , with 32 frames averaged for acquisition of each projection (using an exposure time for each frame of 120ms) (Figures 3.16a-d).

Figure 3.16: Micro CT of a phantom tube

Radiograph of one of the tubes acquired at a single rotation step, and the transmitted intensity (attenuation coefficient) through the entire object. 7b) shows a reconstructed slice of a cross-section through the centre of a tube, revealing the local X-ray attenuation distribution. The colormap is based on a grey level range of 0 to 255 (converted to colour) representing the background (blue) through to the material (red). In order to calculate the volume of material in the walls of each of the tubes, a segmentation method was applied. 7c) This involved thresholding (according to the full width half maximum) the grey level dataset whereby voxels with a grey level value above a certain threshold level were set to 1, representing the polymer wall of the tube, and the remainder, representing the interior of the tube, were set to 0. 7d) shows the same cross-sectional slice but with the threshold applied. The number of voxels labelled as 1 was then calculated and the voxel size (30 m × 30 m × 30 m) used to find the volume of material contained within the tube walls.

a) ***b)*** ***c)*** ***d)***



Measurement of Phantom tube and airway length

RB1 length was measured using the Mimics© software package (Materialise, Belgium). The series of images generated with the Siemens scanner were imported in to the software package using a CT conversion. The conversion increases the contrast in the lung by setting the Hounsfield unit of voxels that were originally in the range [-1024 HU; -824 HU], to the Hounsfield unit of air, i.e. -1024 HU. This conversion can be done without any change in the physical properties of the lung since no human tissue produces a Hounsfield unit in this range.

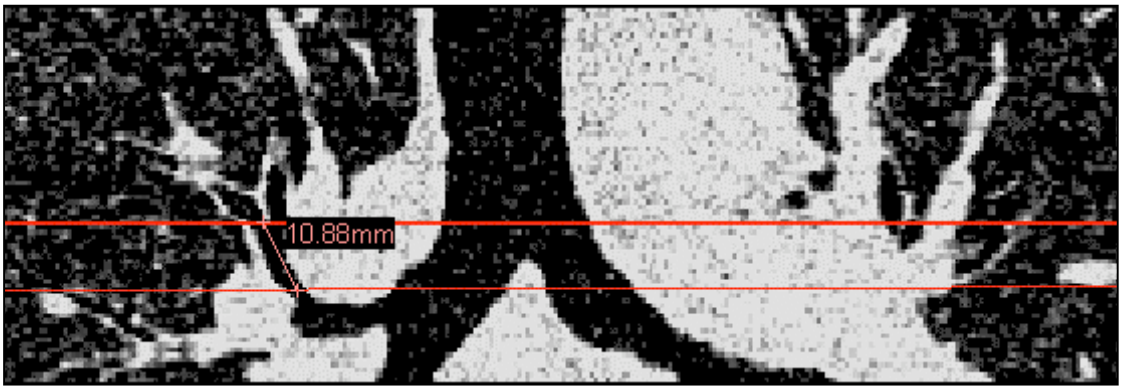
The Mimics© software allowed the limited CT data set to be viewed simultaneously in coronal, saggital and transverse planes. The origin of the RB1 bronchus was first identified in the transverse plane and a marker line drawn across the airway.

The RB1 bronchus was then tracked in the transverse plane until the pre division slice was identified; at this point a second marker line was placed across the airway. The marker lines were identified in the coronal plane and the length of RB1 was measured by connecting the marker lines with a line drawn across the centreline of the airway lumen (figure 3.17). For measurement of phantom tube lengths the tubes were measured across the centre line of the lumen in the coronal section.

The length of the phantom tubes measured using Mimics© was compared to the length of the tubes measured using vernier callipers and micro CT.

Figure 3.17: Measurement of RB1 length using the Mimics© software.

Image of a limited CT scan of the RB1 bronchus reconstructed in the coronal section using the Mimics© software. The length of the RB1 bronchus measured across the two marker lines which were identified in the transverse section is 10.88mm.



Assessment of radiation exposure.

Dosimetry calculations were performed to quantify the effective dose (mSv) of radiation based upon our limited CT scanning protocol and our Siemens Sensation 16 scanner. Effective dose was calculated using the ImPACT CT dosimetry calculator(Version 0.99x; Oxford, UK) with dose distribution data derived using the Monte Carlo approximation and limited scan region estimated based upon the programs mathematical hermaphrodite phantom. We also estimated the Effective dose by using a simplified estimate that minimises method specific difference from reported values, dose not require anatomical localisation to a phantom and can be derived form the simple equation

$E = k \times DLP$[399;400]

Where DLP is the dose length product on the scanner console (Gy*cm) and K is a conversion coefficient ($mSv*mGy^{-1}*cm^{-1}$) that varies according to body region scanned (K: chest =0.014 $mSv*mGy^{-1}*cm^{-1}$). The estimation of E by this method generally varies by <15% of more complex calculations based upon the Monte Carlo approximation[400].

Analysis

All data were expressed, as the mean ± SEM. Accuracy was assessed by Mean % errors of measurements compared to the gold standard stereomicroscope measurement. Inter observer agreement for tube cross sectional measurements was assessed by Pearson's correlation coefficients, Bland Altman plots and the intra class correlation coefficient using a 2 way ANOVA. Repeatability was assessed using intra class correlation coefficients and Bland-Altman plots.

Results;

Window level and window width are both critical in the manual measurement of geometry

A plot of the mean (sem) % error in wall area against the range of window levels and width for all phantom tubes suggested that ideal widow level is between -400&-600HU and that the ideal window width is between 1200&1600HU (figure 3.18a&b).
The window level giving 0% error in wall area was determined for each tube and plotted against the WA (figure 3.18c). This plot demonstrated a bimodal distribution of window level required to produce 0% error in WA. Larger tubes WA >10mm^2 required a smaller window level (mean ± SEM: -910(34) HU), whereas smaller tubes WA < 10mm^2 required a much larger window level –346(51) HU.

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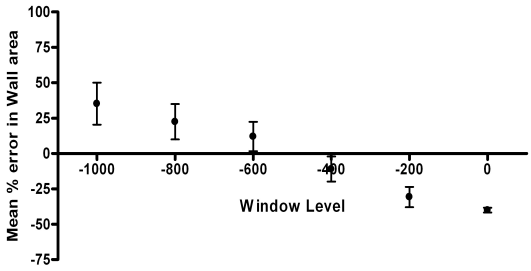
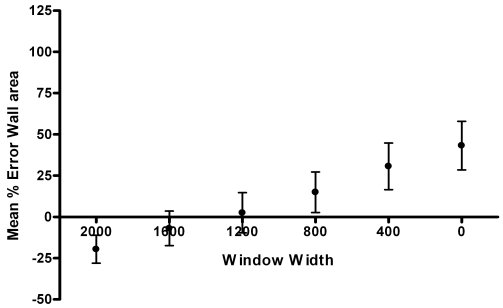
Figure 3.18: Influence of window level (a) and width (b) on manual measures of airway wall area and bimodal distribution of ideal window level according to size of the airway wall.

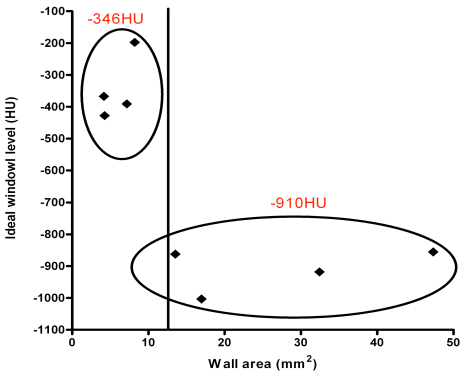
a) Fixed Window Width 1600 HU



b) Fixed Window Level -450 HU



c) Window Width Fixed 1600HU, Ideal Window level giving 0% error



Manual measurement of airway wall geometry is highly repeatable but subject to size dependant error.

There was excellent agreement between observers for measurement of airway dimensions and the observer measurements and the gold standard stereomicroscope measurements. (Table 3.11). Bland-Altman plots comparing the difference between observer measurements and the stereomicroscope vs. the mean of these two measures for LA and WA were performed (Figure 3.19a&b). The mean unsigned % error for tubes 2-9 was TA (4.9%), WA (10.9%), LA (10.5%), WT (10.9%), %WA (8.5%).Bland-Altman plots assessing repeatability showed that within observer measurements were highly repeatable, ICC=1 LA & WA (figure 3.19c&d).

There was a size dependant error in measured total area and wall area that followed a close linear relationship, potentially allowing accurate prediction of %error according to airway size (figure 3.20a&b).

The mean % error in WA was predicted from the windowing analysis for each tube at a WL of –450HU and a WW of 1600HU, this error was then compared to the true mean error measured by the three observers in the manual validation and demonstrated a significant correlation R^2=0.51 $P<0.05$ suggesting that the error in tube measurements is strongly related to window setting

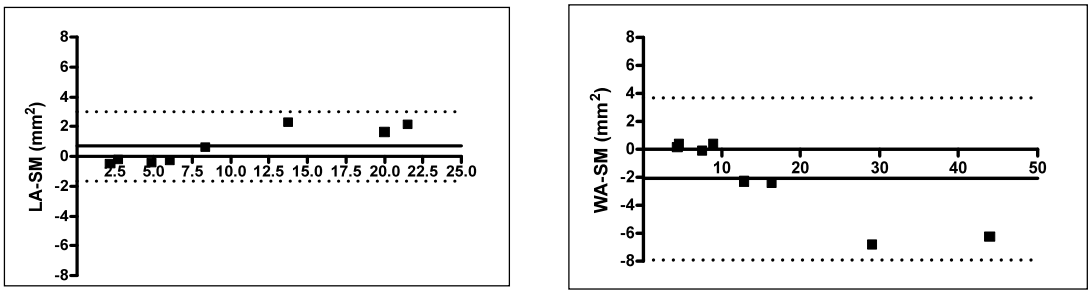
Table 3.11: Repeatability and accuracy of manual measures of tube geometry

Intra class correlation	Interobserver	Observer vs. Stereomicroscope
Wall area	**1**	**0.98**
Luminal area	**1**	**0.99**
Total area	**1**	**1**
Wall thickness	**1**	**0.96**
% Wall area	**0.99**	**0.72**

Figure 3.19: Bland-Altman Plots: Repeatability and accuracy of manual measurements of luminal and wall area.

a&b) Difference between the mean measurements of the 3 observers vs. stereomicroscope (y-axis) against the mean of the observers and the stereomicroscope (x-axis).**c&d)** Difference between the mean of serial measures of two observers (2 weeks apart) of luminal and wall area (y-axis) against the mean of the repeated observations (x-axis). Two dotted and solid lines represent mean±2SD and the mean of the difference, respectively. Luminal area (LA), stereomicroscope (SM), Wall area (WA), Measurement 1 (M1) and Measurement 2 (M2).

a) **b)**



c) **d)**

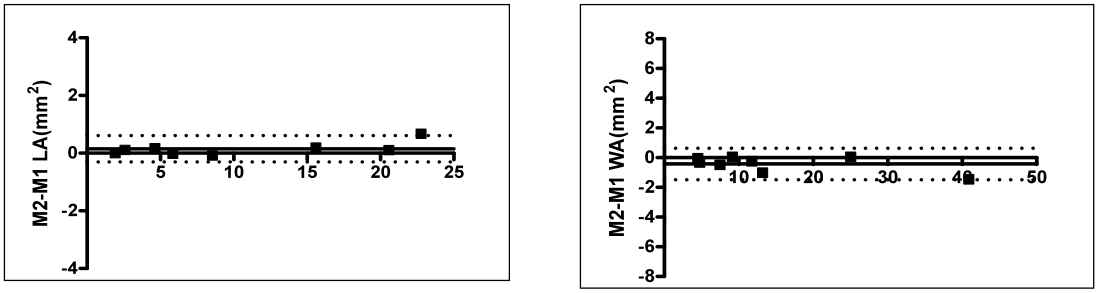
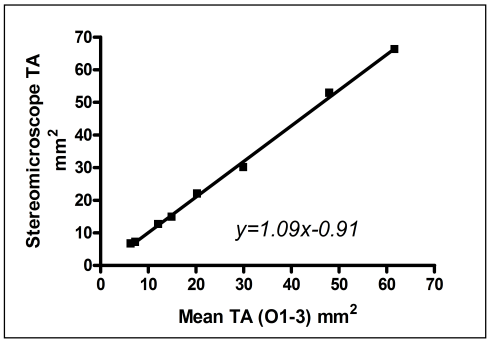


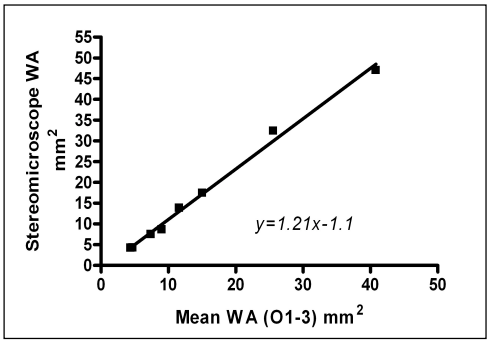
Figure 3.20: Size dependant error in total and wall area with manual validation

a) x-axis: mean total area (TA) of each phantom tube for observers 1-3 (O1-3) and y axis: Stereomicroscope measured gold standard total area. b) x-axis: mean wall area (WA) of each phantom tube for observers 1-3 (O1-3) and y axis: Stereomicroscope measured gold standard wall area.

a)



b)



Full-Width at Half Maximum is an accurate and repeatable measure of airway geometry

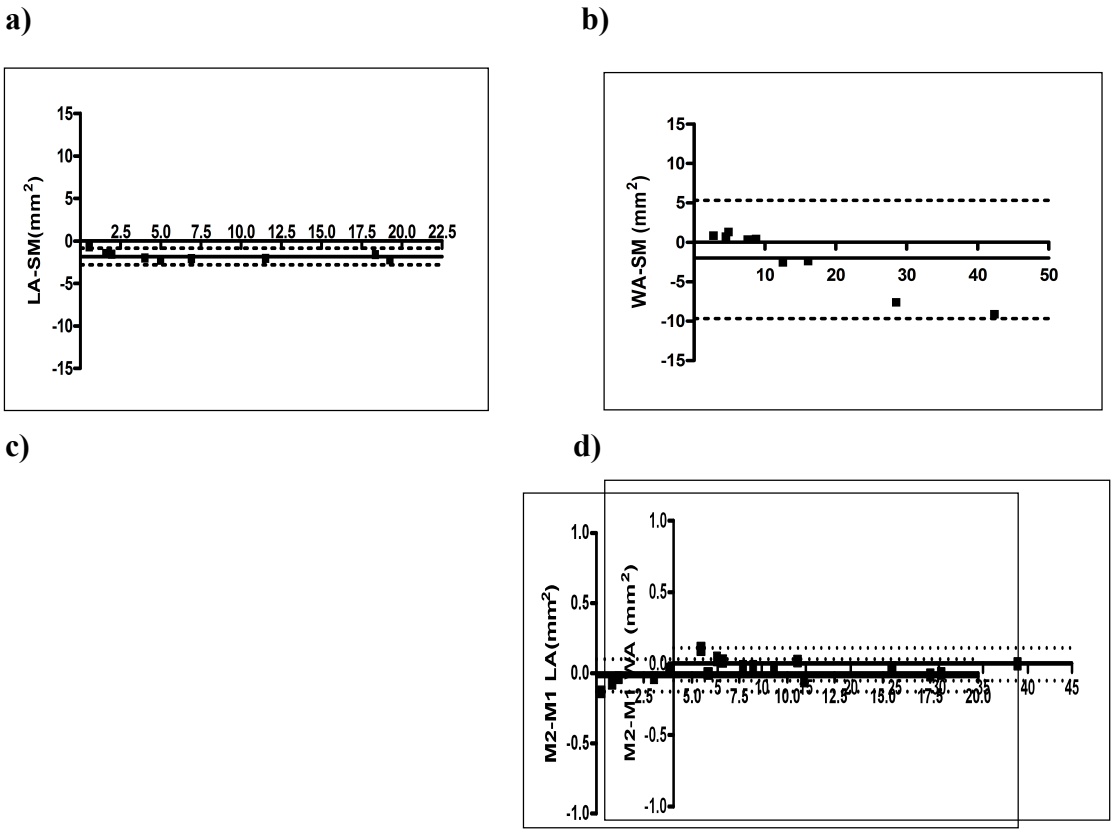
There was excellent repeatability between observers for measurements of WA, LA and TA (T1-9) and also between observers and the stereomicroscope gold standard ICC=1. There was an excellent correlation of airway wall geometry (WA, LA, %WA) at 3 different combinations of slice thickness and collimation (0.75,0.75mm; 0.75,2mm; 2,1.5mm) with an intra class correlation of 1 for all 3 combinations.

Bland-Altman plots comparing the difference between observer measurements and the stereomicroscope versus the mean of these two measures for LA and WA were performed (figure 3.21a&b) and showed evidence of systematic bias as tube size became smaller. Bland-Altman plots assessing repeatability of the algorithm for LA and WA measurements demonstrated that the program produced highly repeatable measures of airway geometry (figure 3.21c&d)

There was an excellent agreement between the three high frequency reconstruction algorithms (B70-F, B80-F, B95UF) for the measurement of tube wall dimensions; WA ICC=0.99; tubes 2-9. The lumen of tube 1 was not visible on the B80f algorithm compared to the B75 and B95 algorithms. The mean±SEM absolute % error in WA measurement (tubes 1-9) was 17.2(2.9)% B70 and 11.4(1.5) B95; $p>0.05$. B95 was significantly more accurate than B70 for the smaller tubes T1-3 with a mean % error of [B95] 7.8(3.8) % vs. [B70] 23.9(4.0)%; $p=0.04$.

Figure 3.21: Bland-Altman Plots: Repeatability and accuracy of FWHM measurements of luminal and wall area.

a&b) Difference between the mean measurements of the 3 observers vs. stereomicroscope (y-axis) against the mean of the observers and the steromicroscope (x-axis). **c&d)** Difference between the mean of serial measures of one observer (2 weeks apart) of luminal and wall area (y-axis) against the mean of the repeated observations (x-axis).Two dotted and solid lines represent the mean±2SD and the mean of the difference, respectively. Luminal area (LA), stereomicroscope (SM), Wall area (WA), Measurement 1 (M1) and Measurement 2 (M2).



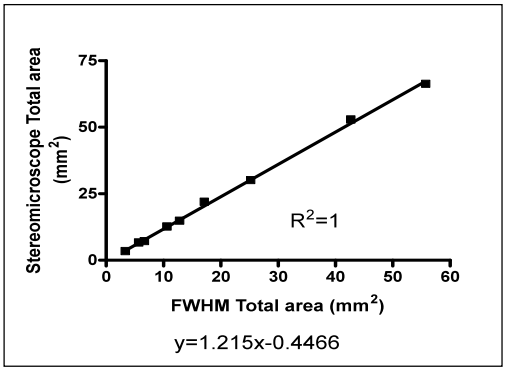
Full-Width at Half Maximum leads to a correctable size-dependant error

Comparison of the total area and WA obtained by stereomicroscopy for the phantom tubes and the FWHM algorithm yielded a near perfect linear relationship which allowed us to derive linear correction equations for wall area and total area (figure 3.22a&b). A similar linear relationship was found for wall area (R^2=0.93) and total area (R^2=0.87) in the sheep *ex vivo* model (figure 3.22c&d). The corrected luminal area and %WA were derived from the corrected WA&TA; LA=corrected TA- Corrected WA and %WA=corrected WA/corrected TA *100.

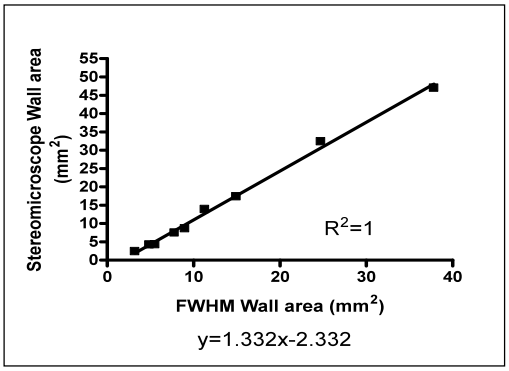
Application of the phantom linear correction equations to the sheep airway data led to a significant reduction in luminal area mean % error (-235(25.5)% to –138(15.2)%; p=0.002).

Figure 3.22: Size dependant error in total and wall area with FWHM method in the phantom (a&b) and sheep airway (c&d) models.

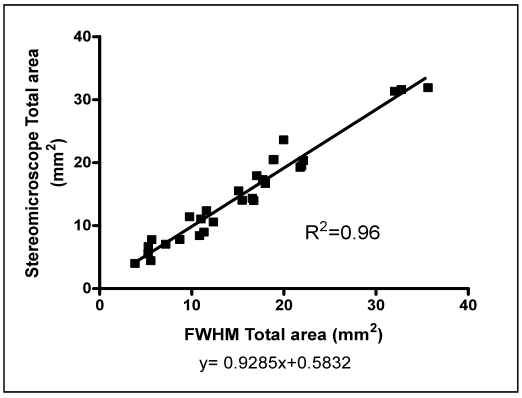
a)



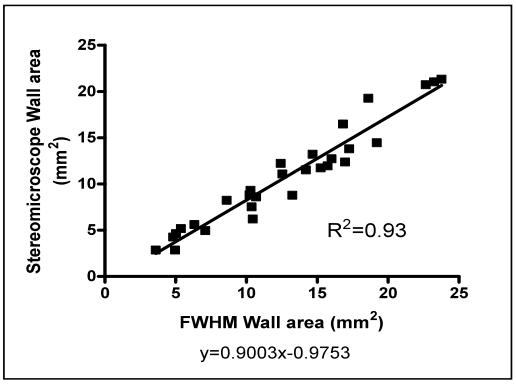
b)



c)



d)



Limit of Accuracy

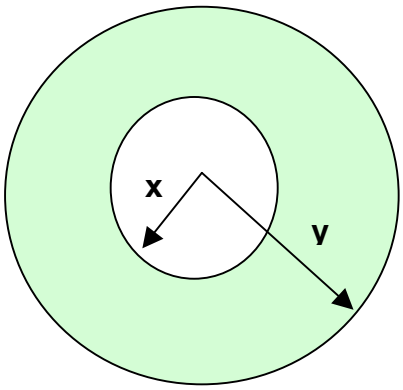
Measurement of the smallest phantom tube T1 resulted in significant error due to partial volume averaging.

The ICC for %WA [tubes 2-9] compared to the stereomicroscope was 0.86 (p=0.01); after applying the linear correction equations to the WA and TA respectively. The mean (SD) unsigned error in corrected %wall area for tubes 2-9 was 4.1(3.2)%.

On the basis of this data we assumed that the limit of CT resolution corresponded to the dimensions of phantom tube 2(TA 6.61mm^2 and radius of 1.45mm) and excluded all airways with a TA of < 6.61mm^2 from airway analysis in the clinical dataset.

Minimum detectable change in luminal radius using FWHM.

We calculated the minimum change in luminal radius detectable based upon a mean % error±2SD in corrected % WA measurements of 10.6% using the FWHM method for tubes 2-9. We assumed that the outer radius is constant at all times in the absence of bronchiectasis and that changes in airway length do not affect cross sectional wall dimensions.



Luminal area (LA)= πx^2

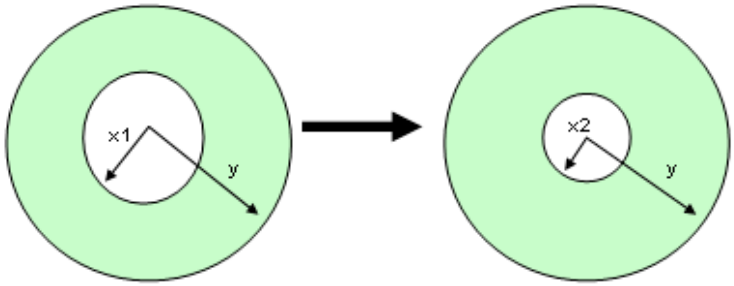
Total area (TA) = πy^2

Wall area (WA) = $\pi(y^2 - x^2)$

%WA = $[(y^2 - x^2)/ y^2] \times 100$

Assuming the outer dimensions of the airway do not change with remodelling – which is probably true in the absence of bronchiectasis- then y is a constant.

Let initial inner radius = x1 and the radius after a period of time = x2.



δ %WA= ([(y^2- $x2^2$)/ y^2] x 100) – ([(y^2- $x1^2$)/ y^2] x 100)

δ %WA =100 ($x1^2$- $x2^2$)/ y^2

4.2%[SD3.2] unsigned corrected mean error FWHM T 2-9

δ %WA > 10.6% unsigned corrected mean error FWHM T 2-9

$x1^2$- $x2^2$ = y^2*10.6/100
multiply by π
δLA = TA*10.6/100
TA Tube 2 = 4.23+2.38 mm^2= 6.61mm^2

ΔLR= 470µm FWHM

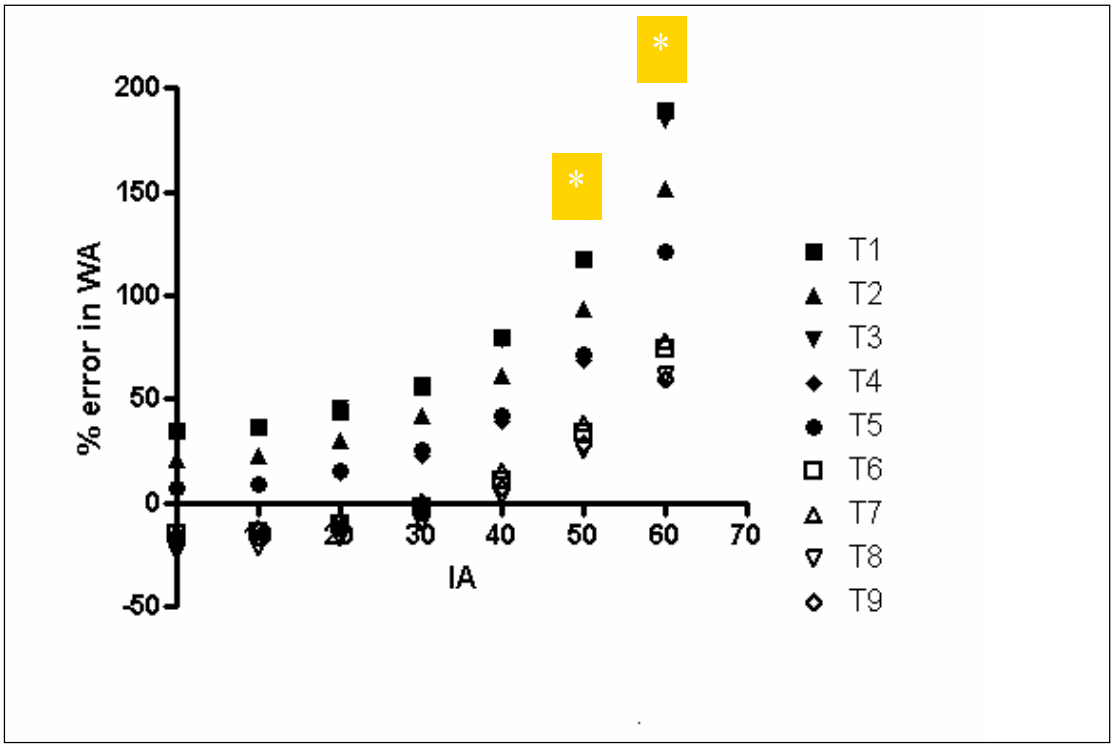
Therefore the minimum serial detectable change in luminal radius using the FWHM method is 470µm.

Oblique orientation is an important determinant of airway geometry

We observed a statistically significant difference in luminal and wall area of the phantom tubes at >/=50º of oblique orientation compared to the stereomicroscope gold standard. We therefore excluded airways with a Maximum/Minimum diameter of >1.5 corresponding to oblique orientation of >48º from the small airway analysis in the clinical dataset (figure 3.23).

Figure 3.23: Oblique orientation and cross sectional error using the FWHM method

% error in wall area (WA) for each phantom tube at an incident angle (IA) of oblique orientation between 0-70º. *p<0.05 vs. stereomicroscope wall area.



Oblique orientation and size dependant error can be corrected

We found that a 3 dimensional plot of the maximum/minimum luminal diameter, measured luminal area/wall area and true luminal area/wall area demonstrated a close parabolic planar fit between plotted points (figure 3.24); best described by the following equations.

True LA= 20-0.014(LA-20)^2 + 3.7 (max/min – 2.1)^2 [R^2=0.85]

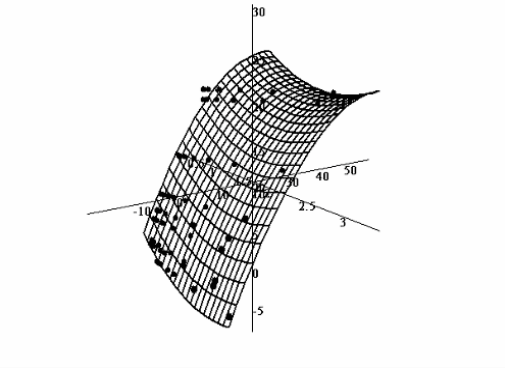
True WA= 50+7.5(max/min-2.3)^2-0.0073(WA-92)^2 [R^2=0.80]

Comparison of cross sectional geometry using micro CT and stereomicroscopy

There was a close correlation between micro CT and stereomicroscopy of the leading face of phantom tubes 1-9 (table 3.12) for the wall thickness (r^2=0.99) and luminal area (r^2=0.99). The mean (sem) unsigned % difference in wall area and luminal area between stereomicroscopy and micro CT was 6.44(1.0) % and 9.70(3.53) % respectively.

Figure 3.24: 3D plot of LA/WA, True LA/WA and maximum/minimum radial diameter for 9 phantom tubes reconstructed from 0-60° with parabolic best-fit plane.

a) Luminal area



b) Wall area

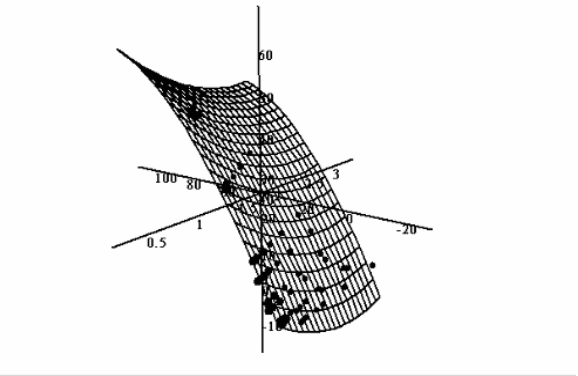


Table 3.12

Comparison of cross sectional wall thickness and area using micro CT and stereomicroscopy of the phantom tubes at the leading face.

Tube	Micro CT Mean Wall thickness (mm)	Stereomicroscope Wall thickness (mm)	Micro CT Mean Luminal area (mm^2)	Stereomicroscope Luminal area (mm^2)
1	0.42	0.47	1.37	0.95
2	0.59	0.58	2.71	2.38
3	0.52	0.56	2.97	2.78
4	0.67	0.73	5.39	5.07
5	0.71	0.77	6.21	6.16
6	0.97	1.04	7.90	8.04
7	1.01	1.09	13.79	12.57
8	1.49	1.55	21.40	20.42
9	2.07	2.12	20.95	19.17

The measurement of phantom tube length using the Mimics© software is accurate and repeatable.

The measurement of phantom tube length using the Mimics© software were highly repeatable with an ICC of 0.92; $p<0.0001$ for the comparison of micro CT, Mimics© and vernier callipers for tube length.

Limited CT of the apical bronchus is a low radiation procedure and could be used to assess airway wall remodeling longitudinally.

Using the Monte Carlo approximation the effective radiation dose from a single limited CT was 0.42mSv. The effective dose based upon the approximation from the scanner console derived dose length product was (32*0.014) =0.45 mSv. This approximates to 30% of the effective radiation dose of a standard full high resolution CT (table 3.13).

We estimate that a single limited CT is equivalent to 24 non-CR CXRs of radiation and is approximately $1/6^{th}$ of the annual background radiation from natural sources in the UK.

Table 3.13: Radiation risk associated with limited CT.

Multislice Protocol	**$CTDI_{vol}$ (mGy)**	**DLP (mGy cm)**	**Effective dose [E] (mSv) [CXRs]¶**
Staging CT lung cancer ^^	10	580	7.25 [381]
HRCT ^^	7	170	2.38 [125]
RB1 bronchus limited CT #	3.92	32	0.45 [24]
CXR ^^	**-**	**-**	0.019
Annual natural Background radiation U.K (NRPB)			2.6

^^Based upon rounded 75th percentile of dose distribution; U.K 2003 survey of doses from CT examinations[401].¶ CXR=Chest x ray. CTDI= CT dose index. DLP= dose length product. E=*K**DLP, K=0.014 mSv*mGy $^{-1}$*cm^{-1}[400].# Limited CT protocol (Glenfield Hospital, UK) 50 mAs, 120 KV, 16*0.75mm collimation, Table feed/rotation 13.5, scan length of 5.3cm [aortic arch to 1cm below carina].

Discussion

We have determined the accuracy and repeatability of cross sectional measures of airway geometry using a phantom /sheep airway model and validation with stereomicroscopy and micro-CT.
We have demonstrated that manual measures of airway geometry are subject to significant size dependant errors that can be predicted and corrected. Furthermore the measurement of airway wall area using manual methods was highly dependent upon the window level as previously described[68;397;402]. In addition our data suggests that window width is also an important determinant of the manual assessment of airway wall area. We found that the there was a bimodal distribution for the ideal window level to measure larger tubes modelling airways with a wall area > $10mm^2$ and smaller tubes with wall area < $10mm^2$, suggesting that the traditional notion of an ideal window width and level of 1600HU and -450 HU for all airways is inaccurate. Nonetheless we demonstrated that manual measures of geometry were highly repeatable between observers at a window level of -450HU and window width of 1600HU and furthermore the error predicted from the windowing analysis at these window settings for the phantom tubes closely correlated with the mean error of the three observers.

We determined the accuracy and repeatability of airway geometric measurements using the full width at half maximum method[392] using a custom made program. We have demonstrated that similarly to manual measures of geometry, the FWHM method leads to significant size dependant error as airways became smaller in both the phantom and sheep airway model. The CT scan measurements using FWHM consistently overestimated the wall area, while underestimating the luminal area in both the phantom and sheep airway models. These errors are due to a variety of factors including the limited spatial resolution of the scanner and the ability of the scanner to detect edges (point spread function). The error could be accurately predicted and corrected. We demonstrated that application of the phantom linear correction equations to the sheep airway measurements led to a significant reduction in error. Finally we have shown that using the phantom linear correction equations the limit of CT resolution equates to a

total airway area of 6.61 mm^2 and radius of 1.45mm and that airways larger than this could be accurately and repeatably measured to within 5% absolute cross-sectional error.

The sheep airways were derived from cut sheep slabs that were snap frozen and imaged using CT followed by measurement of airways using stereomicroscopy. It is likely that there would have been some tissue shrinkage prior to stereomicroscopic measurements which were performed approximately 3-4 hours after the CT scan, this would have led to an even greater error in the measurement of geometry using the FWHM method. We therefore adopted the phantom linear correction equations to correct cross sectional error in clinical studies on the premise that the phantom model did not have the confounding effect of tissue shrinkage which would reduce the accuracy of the stereomicroscope gold standard measurements of geometry.

We found that the intraobserver repeatability was significantly superior for the FWHM algorithm compared to simple manual measurements. In contrast both manual methods and the FWHM method had similar accuracy when compared to the gold standard stereomicroscopic measurements. On the basis of superior repeatability and the ease of using an automated algorithm to measure airway geometry we decided to used the FWHM algorithm to measure airway geometry in clinical studies. A further advantage of the automated algorithm is that it defines airway geometry independent of window level and width as the algorithm uses the full HU range of attenuation values, therefore eliminating the need to predetermine window settings.

We found that reconstruction kernel (B70-F, B80-F, B95UF), slice thickness (0.75-2mm) and collimation (0.75-2mm) did not significantly influence the measurement of tube geometry using the FWHM method, however narrow collimation and slice thickness did significantly improve the accuracy of the measurements of smaller tubes (data not shown). We therefore adopted a narrow collimation (0.75mm) protocol for our clinical studies.

In view of the pressing need for longitudinal studies of airway remodeling in asthma, we sought to determine the minimum serial detectable change in luminal radius over time using FWHM method based upon the limit of detection and repeatability of our validation. We assumed that the outer radius of the airway was a constant (in the absence of bronchiectasis). Our data suggests that the minimum serial detectable change in luminal radius is 470 microns using the FWHM algorithm. This would suggest that current CT technology is unable to detect structural change of individual structural components within the airway wall over time as this is likely to occur over a log fold lower magnitude.

As suggested by previous investigators we found that oblique orientation of the phantom tubes led to a significant error in cross sectional geometry[403]. The error became significant compared to the stereomicroscope gold standard measurements when the angle of oblique orientation exceeded 50°. We therefore excluded airways with a maximum/minimum diameter of > 1.5 corresponding to oblique orientation of > 48° from analysis in clinical studies imaging the RB1 bronchus and smaller airways.

In view of the fact that we anticipated performing clinical studies using archived HRCT scans which would only capture the RB1 bronchus in a single slice that may have been relatively oblique in orientation we attempted to correct for both size dependant error in geometry and oblique orientation using the FWHM method. We were able to find close parabolic planar fits to 3 dimensional plots of true luminal/wall area, measured maximum/minimum luminal diameter, and measured luminal/wall area.

We sought to compare the gold standard stereomicroscopic measurements of airway wall and luminal area with micro-CT. The resolving power of micro CT is far superior to conventional macro CT with a voxel size in the micron order of magnitude[394]. We deliberately chose an aluminium filtered micro-CT system to minimise the observe effect of beam hardening that we had seen with conventional CT.

We found an excellent correlation between Micro CT and stereomicroscope wall geometry.

A major concern with imaging studies in asthma is the radiation dose accrued by the patient[391]. We calculated the radiation dose using a commercial dosimetry program based upon the Monte Carlo approximation. We determined that the effective radiation dose from a single limited CT scan of the RB1 bronchus was between 0.42-0.45mSv which is less than 30% of the effective dose of a traditional HRCT scan in the UK, 1/6th of the annual natural background radiation from natural sources in the UK and approximated 24 chest x ray equivalents. Therefore our limited CT protocol is a low dose protocol that may be used serially to assess remodeling in adult asthma.

Our validation has a number of limitations, the phantom model tubes had a higher attenuation spectrum compared to true airway tissue. The sheep airways were derived from cut sheep slabs that were snap frozen and imaged followed by measurement of airways using stereomicroscopy and therefore would have had limited correlation with inflated human lung tissue.

In conclusion we have determined the accuracy and repeatability of the FWHM algorithm to measure airway geometry and derived correction equations to minimise cross sectional error in volumetric CT scans and error due to cross sectional airway size and oblique orientation in non volumetric HRCT scans.

3.2.2

Airway wall geometry in asthma and non-asthmatic eosinophilic bronchitis.

Abstract

Background. We sought to identify whether there are differences in airway geometry in asthma versus Non Asthmatic Eosinophilic Bronchitis (EB). **Methods.** 12 subjects with mild-moderate asthma, 14 subjects with refractory asthma, 10 subjects with EB and 11 healthy volunteers were recruited. Subjects with asthma and EB received a 2-week course of oral prednisolone (0.5mg/kg) accompanied by limited CT scans of the apical segmental bronchus; right upper lobe (RB1) before and after treatment. Narrow collimation (0.75mm) CT scanning was performed from the aortic arch to the carina. **Results.** Mild-moderate and refractory asthma were associated with RB1 wall thickening in contrast to subjects with EB who had maintained RB1 patency without wall thickening (mean [SD] % wall area and luminal area mild-moderate asthma 67.7[7.3]% and 6.6[2.8] mm^2/m^2, refractory asthma 67.3[5.6]% and 6.7[3.4] mm^2/m^2, healthy control group 59.7[6.3]% and 8.7[3.8] mm^2/m^2, EB 61.4[7.8]% and 11.1[4.6] mm^2/m^2 respectively; $p<0.05$). Reduced luminal patency and airway wall thickening of non RB1 airways was a feature of asthma only. Proximal airway wall thickening was associated with AHR in asthma ($r=-0.56;p=0.02$). **Conclusions.** Maintained airway patency in EB may protect against the development of AHR, whereas reduced patency may promote AHR in asthma.

Introduction

Asthma is characterised by typical symptoms, variable airflow obstruction and airway hyperresponsiveness (AHR) in association with airway inflammation and structural changes within the airway wall, collectively known as remodeling[284]. In bronchial biopsies from asthmatics, remodeling of different components of the airway wall including the vasculature, smooth muscle, glands, matrix; and the influx of CD8+ T-cells correlate with airflow obstruction[76;368] and FEV_1 decline[350]. In contrast the association between AHR and remodeling is unclear.

Mathematical models suggest that thickening of the airway wall is fundamental in the development of AHR in asthma[290] and underpins the view that AHR is a consequence of remodeling in response to chronic inflammation[386]. Intriguingly, Niimi et al have demonstrated that airway reactivity is inversely proportional to the thickness of the RB1 bronchus (apical segmental bronchus of the right upper lobe)[67]. The RB1 bronchus is a useful marker of remodeling in asthma[68] and remodeling of this airway correlates with distal airway wall thickening and airflow obstruction in asthma and COPD[65;69;96]. Therefore, in some circumstances remodeling may protect against AHR. Possible explanations for this apparent paradox is that remodeling and AHR may be epiphenomena or alternatively remodeling may initially exacerbate AHR, but in persistent disease may stiffen the airways protecting against AHR[287].

Non-asthmatic eosinophilic bronchitis (EB) is a powerful disease control model to study potential mechanisms of AHR. It accounts for approximately 10% of referrals to a specialist cough clinic[310] and is characterised by eosinophilic airway inflammation demonstrated by sputum, bronchoalveolar lavage and bronchial biopsy, without any evidence of variable airflow obstruction or AHR[105;314;316;364]. We have demonstrated that vascular remodeling[368] and thickening of the reticular basement membrane[105;314] occur to a similar degree in asthma and EB and it is therefore unlikely that these components of the remodeling process contribute to AHR in asthma. Park *et al* have recently reported that airway wall thickening of proximal airways was not a feature of EB[318], but confidence in this conclusion is undermined by the lack of standardisation of

airway measurements for subject size and the short duration of disease of 5-8 months[319], questioning whether these subjects had sufficient time to develop remodeling. Therefore whether airway remodeling is a feature of EB remains uncertain.

We hypothesised that remodeling is a feature of EB and asthma and that AHR and remodeling are independent. We sought to 1) identify whether there are differences in the static geometry of the RB1 bronchus a proximal 3rd generation conducting airway and non RB1 airways (generations 3-6) between asthma and EB and 2) whether these differences may account for the observed absence of lower airway hyper responsiveness in EB. 3) In addition we sought to establish whether there are changes in the static geometry of the RB1 bronchus after a 2 week prednisolone trial in patients with mild to moderate persistent asthma and EB.
To test our hypothesis we studied subjects with EB, mild-severe refractory asthma, and healthy controls.

Methods

Subjects

12 subjects with mild to moderate asthma (GINA treatment severity 1-4), 14 subjects with refractory asthma, 10 subjects with EB and 11 healthy volunteers were recruited prospectively from Glenfield Hospital outpatients, staff and by local advertising. One of the patients with mild to moderate asthma withdrew after the baseline CT scan for non medical reasons. All subjects were non-smokers with a smoking history of <10 pack years; had been free of exacerbations and on stable treatment for 8-weeks prior to entry into the study. EB was defined according to the ACCP criteria[364], all patients had a history of cough and evidence of a sputum eosinophilia of > 3% without lower airway responsiveness (methacholine PC_{20}>16mg/ml) or symptoms of variable airflow obstruction. In addition patients with EB had minimum disease duration of 5 years without evidence of airflow limitation during this period. Asthma was defined by one of more of the following objective criteria: significant bronchodilator reversibility of >200mls, a provocation concentration of methacholine causing a 20% fall in FEV_1

(PC_{20}) of less than 8mg/ml or a peak flow amplitude % mean over 2 weeks of more than 20%. Asthma severity was classified using the Global Initiative for Asthma (GINA) guidelines[10] treatment steps and refractory asthma was defined according to the American Thoracic Society consensus[9]. Normal subjects had no history of respiratory disease and normal spirometry and PC_{20}. The Leicestershire ethics committee approved the study and all subjects gave their written informed consent.

Table 3.14: Clinical Characteristics

	Control (n=11)	Mild to Moderate asthma (n=12)	Refractory asthma (n=14)	Eosinophilic Bronchitis (n=10)
Age (yrs)	60.1(10.4)	46.3(18.0)	51.3(8.1)	57.1(12.3)
Sex M:F	7:4	8:4	9:5	5:5
Atopy (%)	36	58	57	60
Disease duration (yrs)	NA	22.8(17.0)	21.7(16.9)	10.6(10.9)
BMI (kg/m^2)	25.4(3.6)	27.9(3.6)	29.1(7.4)	26.5(6.4)
BDP equivalent	0	692(748)	1829(760)	1460(776) *¶
Oral CS	0	0	9.6(4.7):14/14	0
Total IgE (KU)#	20[9-44]	106[36-314]	169[72-395]	116[56-242]*γ
Peripheral blood eosinophils #	0.14[0.08-0.24]	0.16[0.1-0.25]	0.27[0.16-0.44]	0.29[0.17-0.47]
FEV_1 % post bronchodilator	106.1(13.8)	87.4(21.6)	79.9(29)	108.4(15.4)*∞
FEV_1/FVC	75.4(6.2)	72.4(10.4)	65.5(22.5)	79.8(10.3)
PC_{20}(mg/ml) #	>16	0.26[0.1-0.72]	2.9[0.17-48]	>16*
Sputum eosinophils % #	0.42[020-0.88	1.2[0.31-4.3]	5.6[1.7-18.1]	4.0[1.2-13.3]*φ
Sputum neutrophils %	67.8(26.9)	49.1(31.3)	55.4(30.5)	52(24.0)
ENO #	16.5 [12.4-21.0]	20.5 [12.5-33.7]	31[15.8-62.0]	34.5[22.7-53.6]

*Data expressed as mean (SD); #Geometric mean [95%CI], Beclamethasone Diproprionante (BDP) equivalents; fluticasone 2:1, Budesonide 1.25:1, Mometasone 1.25:1. CS=corticosteroid, ENO= exhaled nitric oxide, BMI= body mass index. Intergroup comparisons: t-test and one-way ANOVA with Bonferroni correction for multiple comparisons.*p<0.05 One Way Analysis of Variance ¶ p<0.05 mild to moderate persistent asthma vs. refractory asthma. ∞ EB/control vs refractory asthma. Φ EB/refractory asthma vs. control. γ EB/refractory/mild to moderate asthma vs. control.*

Protocol and clinical characterisation

All subjects underwent clinical characterisation including spirometry, allergen skin prick tests for common aeroallergens, measurement of exhaled nitric oxide (eNO) concentration[404], a methacholine inhalation test[241], sputum induction[210] and peripheral blood eosinophil count and total IgE.

CT scanning protocol

All subjects had a limited CT scan of the RB1 bronchus. CT scanning was performed with a Siemens Sensation 16 mutislice scanner. Scans were obtained at 16x0.75mm collimation, 120 kV, 50 mAs with a table speed of 13.5 per 0.5 second scanner rotation. Images were reconstructed at 0.75 mm spacings using 295 mm (139mm for phantom datasets) field of view with a 512 x 512 matrix and a very sharp reconstruction algorithm (B70-f). CT scans were performed at full inspiration and patients were instructed to complete an adequate breath hold prior to scanning. All patients with severe asthma and 5/11 patients with mild to moderate persistent asthma were on maintenance treatment with a long acting β-agonist (LABA) which was taken 2hrs prior to their CT scan. 6/11 patients with mild to moderate persistent asthma were not on a LABA and a short acting bronchodilator was not administered prior to the CT scan to compare the effects of increased ASM tone against bronchodilator use prior to CT scanning in mild to moderate persistent asthma. All CT scans had been reported as normal by an experienced radiologist prior to analysis.

Subjects with mild-moderate asthma or EB had a limited CT before and after 2-weeks of treatment with prednisolone 0.5mg/kg once daily. Healthy controls and severe refractory asthmatics underwent a single CT either without treatment or after 2-weeks of prednisolone respectively. CT scanning was performed within 1 day of the prednisolone trial and clinical characterisation was performed within 5 days of the prednisolone trial.

Validation of airway geometry.

Airway geometry was analysed using the full width half maximum (FWHM) technique with a custom program (Emphylyx-J V 1.00.01; British Columbia University, Vancouver)[398]. A phantom airway model and *ex vivo* sheep airway model were used to validate the airway algorithm and linear correction formulae were derived to correct the observed overestimation of wall area (WA) and under estimation of luminal area (LA) as airway size became smaller. Based upon the validation airways all airways that had a maximum/minimum diameter of >1.5 were assumed to be significantly oblique and excluded from analysis. All airways smaller than 6.61 mm^2 total area (uncorrected for body surface area) corresponding to the limit of accuracy were also excluded from airway analysis (see previous CT validation section).

Airway cross sectional analysis

All airways visible on the CT images, including RB1 were identified and measured using the full-width at half maximum algorithm by two observers blinded to clinical characteristics. The RB1 bronchus was tracked from its origin to its point of division and measurements were taken at 0.75mm intervals[67;68]. The mean of the measurements of WA, LA were corrected for body surface area (BSA)[405]. The total area (TA) and percentage wall area (%WA) were derived from the LA and WA.

All other visible airways (generations 3-6) were measured and corrected for BSA. TA and WA were corrected using the linear correction algorithm derived from the phantom airway model and the LA, % WA and LA:WA ratio (a marker of luminal patency relative to the airway wall) were derived from the corrected WA&TA as described in the phantom model.

After correction for size dependant error and exclusion of oblique airways the total number of non RB1 airways analysed was 386 across the 4 groups. The mean(SD) number of non RB1 airways measured in each patients was 8.5(3.7).The median[IQ

range] of the total airway area corrected for body surface area was 7.9[5.5-10.4] mm^2/m^2, and the mean (SD) Maximum/Minimum luminal diameter (SD) was 1.3(0.14).

RB1 length.

RB1 length was measured using the Mimics© software package (Materialise, Belgium)[see previous CT validation section].

Analysis.

To have an 80% power at the 5% level to detect a difference between groups in %WA of 7.5% assuming a SD of 6%[67;68], we estimated that 10 subjects would be required in each group

Statistical analysis was performed using PRISM Version 4 (Prism, San Diego, Calif) and regression analysis using SPSS version 13.0 (SPSS Inc, Chicago, III). Normally distributed data were expressed as mean (SD), log normally distributed data was described as geometric mean (95% CI) and non-normally distributed data were described as median (range). One-way analysis of variance with Bonferronis correction (normally distributed data) and Kruskal-Wallis with Dunns intergroup comparison for non-normally distributed data, were used to compare multiple groups. Student's t-tests and Chi-squared tests were used for between group comparisons (two groups) and categorical data respectively. Bland-Altman plots were used to assess agreement between observers for airway measurements. A p value of <0.05 was taken as statistically significant.

Results

The FWHM method is a repeatable measure of airway wall geometry.

Measurements of the %WA of the RB1 bronchus were highly repeatable between observers r_s=0.8; p<0.0001 (n=53).

Prednisolone attenuated inflammation, but did not affect airway wall geometry

The airway geometry of RB1 assessed by CT in the subjects with mild-moderate asthma or EB was not significantly different before or after treatment with oral corticosteroids. In contrast, sputum and blood eosinophil counts in asthma and sputum eosinophils and ENO in EB were attenuated (Table 3.15).

Asthma is characterised by proximal airway wall remodeling compared to healthy controls.

The mean (SD)% WA of RB1 post-prednisolone was significantly greater in mild to moderate persistent asthma [67.7 (7.3)%] and refractory asthma [67.3 (5.6)%] compared to the control group [59.7 (6.3)%] but not to the EB cohort [61.4 (7.8)%]; p=0.01 (Figure 3.25a-c& 3.26a).

Eosinophilic bronchitis is characterised by maintained airway patency of the RB1 bronchus compared to asthma.

The mean (SD) luminal area (mm^2/m^2) of the RB1 bronchus post-prednisolone was significantly greater in EB 11.1(4.6) compared to subjects with mild to moderate asthma 6.6(2.8) and refractory asthma 6.7(3.4) but not to the control group 8.7(3.8); p=0.02. Similarly the mean (SD) ratio of the luminal: wall area of RB1 post-prednisolone was significantly smaller in moderate persistent asthma [0.49 (0.17] and refractory asthma [0.50(0.13)] compared to the control group [0.69(0.18)] but not to the EB cohort [0.65 (0.20)]; p=0.01. (figure 3.25a-c&3.26b&c)

Table 3.15: The effect of 2 weeks of prednisolone on measures of inflammation and airway geometry in asthma and EB.

	Mild to Moderate asthma (n=11)		**EB (n=10)**	
Inflammation	**Pre**	**Post**	**Pre**	**Post**
Sputum eosinophils %#	1.15[0.3-4.3]	0.66[0.2-1.8]	4[1.2-13.3]	1.2[0.5-2.8]*
Blood eosinophils #	0.17[0.1-0.27]	0.06[0.03-0.13]*	0.29[0.18-0.47]	0.18[0.1-0.33]
ENO 50 mls/s (ppb)#	20.4[11.8-35.3]	18[12.1-26.8]	34.9[22.7-53.6]	22.6[15.6-32.7]*
RB1 Geometry	**Pre**	**Post**	**Pre**	**Post**
WA/BSA	12.1(3.0)	13.16(2.7)	16.14(6.3)	17.08(4.6)
LA/BSA	6.6(3.4)	6.56(2.8)	10.99(5.1)	11.05(4.6)
TA/BSA	18.67(6.2)	19.73(5.0)	27.12(10.3)	28.12(8.6)
%WA	66.4(7.5)	67.7(7.3)	60.0(7.8)	61.4(7.8)

Mean(SD); paired t-tests for pre and post prednisolone comparisons for RB1

 # Geometric mean [95% CI].

WA=wall area, LA=luminal area, TA= Total area, BSA= Body surface area.

**p<0.05 post prednisolone vs. pre prednisolone*

Geometric measurements in mm^2/m^2

Figure 3.25a-c: CT images of the RB1 (x5 magnification).

Post-prednisolone images illustrating a) a healthy control with an absence of airway wall thickening and preserved luminal calibre, b) a refractory asthmatic with airway wall thickening and narrowing of the airway lumen and c) a subject with EB with maintained patency of the airway lumen without evidence of wall thickening.

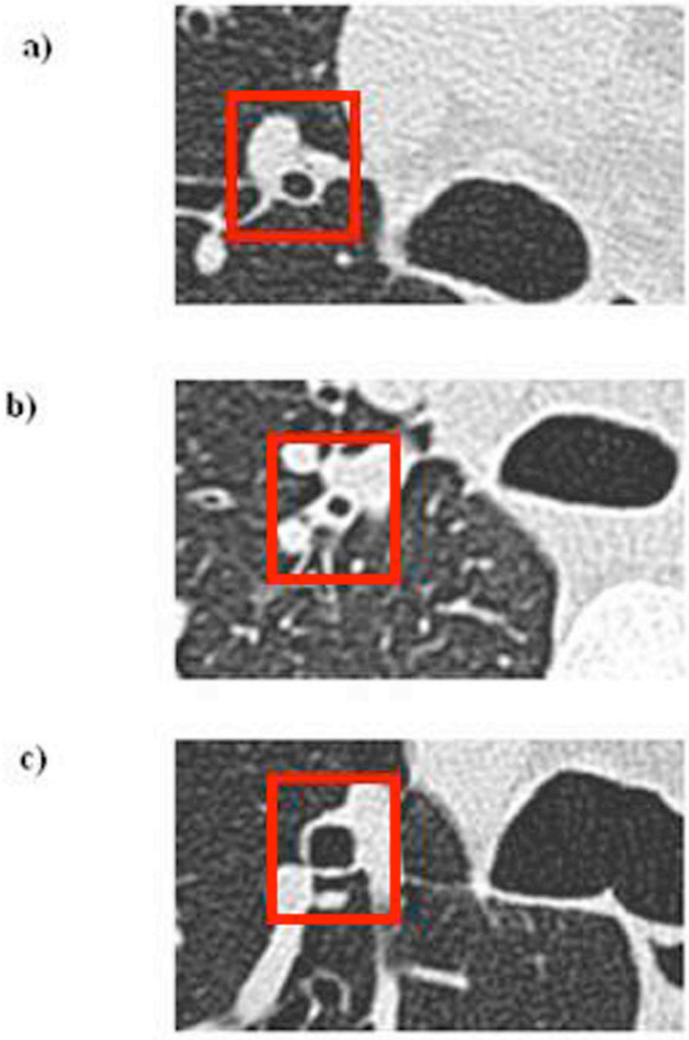
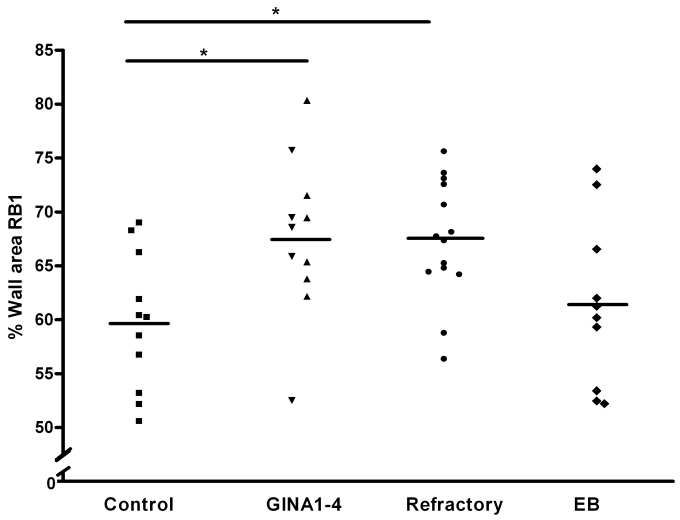


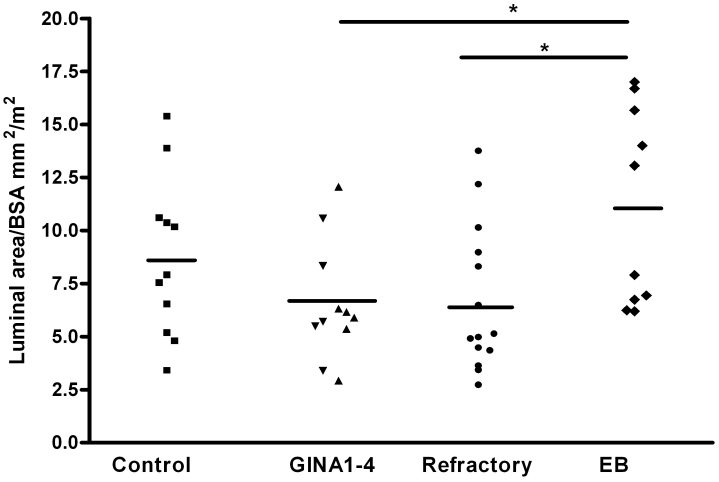
Figure 3.26: Reduced luminal patency due to airway wall thickening in asthma but not EB

Mean %WA (a), luminal area/BSA (b), luminal: wall area ratio (c) and length (d) of RB1 (right upper lobe ASB) post-prednisolone in subjects with asthma, EB and healthy controls. ▼ *Took a LABA pre CT,* ▲ *Did not take a LABA pre CT. *p<0.05*

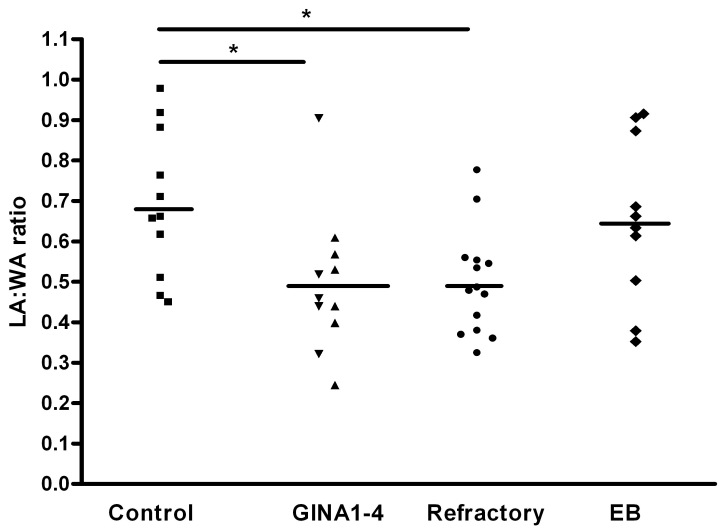
a)



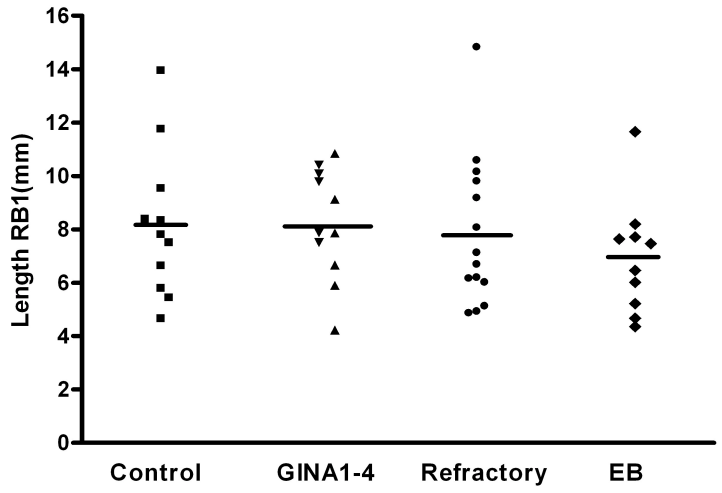
b)



C)


*
*
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
LA:WA ratio
Control
GINA1-4
Refractory
EB

d)


16
14
12
10
8
6
4
2
0
Length RB1(mm)
Control
GINA1-4
Refractory
EB

Airway shortening of RB1 is not a feature of asthma or EB

The measurement of airway length of RB1 was highly repeatable between observers (r_s=0.86; p<0.0001), mean bias (95% CI) 0.94 [0.6-1.3]mm and within observer (r_s=0.8; p<0.0001), mean bias 1.0 [0.6-1.4]mm. The mean (SD) length of the RB1 bronchus was similar in all groups; 8.2(2.9)mm controls, 8.2(2.1) mild to moderate persistent asthma, 7.8(2.8) mm refractory asthma and 6.9(2.1) mm in EB; p=0.63(figure 3.26d).

Airway luminal patency, % wall area and RB1 length were not related to the use of a long acting bronchodilator prior to CT scanning in patients with mild to moderate persistent asthma.

The mean (SD) %wall area (66.42(8.6) % vs. 68.8(6.7) %; p=0.62), luminal area/BSA (6.70(2.8) vs. 6.46(3.0) mm^2/m^2; p=0.89) and RB1 length (9.1 vs. 7.4 mm; p=0.19) did not differ significantly in patients that had taken a long acting bronchodilator prior to CT imaging compared with those that had not post prednisolone.

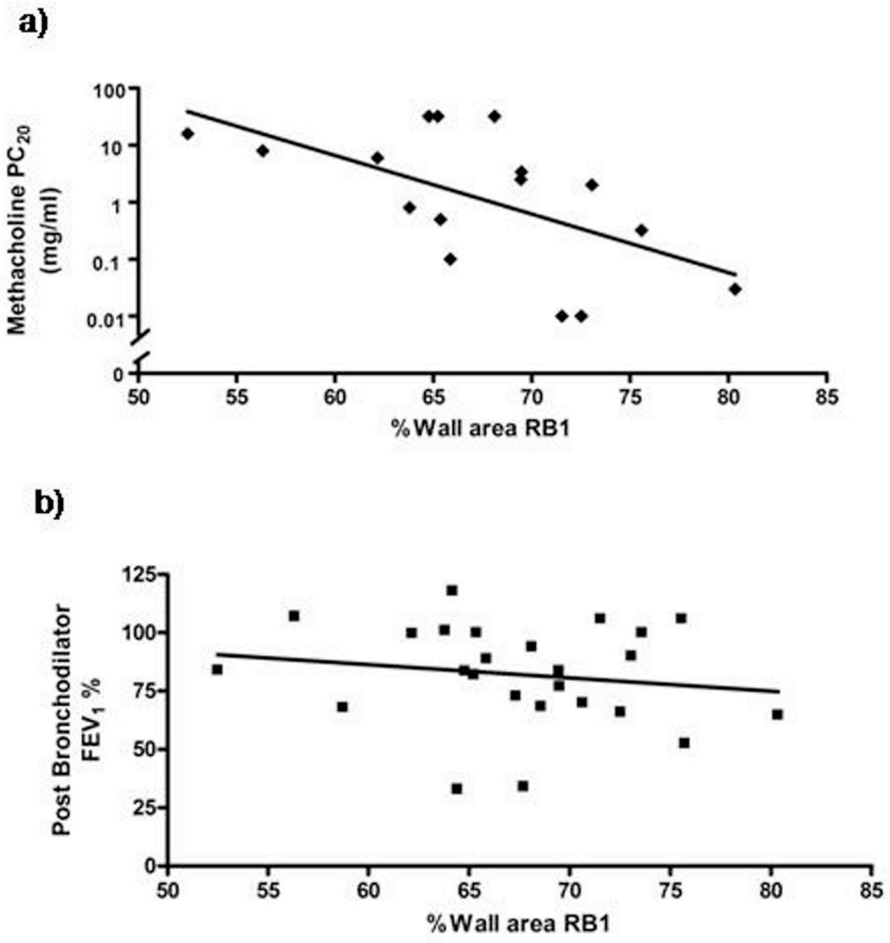
% Wall area of the RB1 bronchus correlates with airway hyper responsiveness in asthma.

The % wall area of RB1 demonstrated a significant inverse correlation with the log methacholine PC_{20} in the pooled asthma cohort post prednisolone (r=-0.56; p=0.02) but not the bronchodilator response % FEV_1 (-0.16; p=0.44), post bronchodilator FEV1 % (r=-0.16; p=0.44) or log sputum eosinophils (r=-0.09; p=0.69) (figure 3.27).

Figure 3.27: Correlation of RB1 thickening in asthma and AHR/airflow limitation.

a: *Correlation of % wall area of RB1 and methacholine PC_{20} in the pooled refractory and mild to moderate asthma cohort.* ***r= -0.56; p=0.02.***

b: *Correlation of % wall area of RB1 and post bronchodilator FEV_1 % predicted in the pooled refractory and mild to moderate asthma cohort.* ***r= -0.16; p=0.44.***



% Wall area of RB1 correlates with mean % wall area of non RB1 airways (generations 3-6).

There was significant correlation between the % wall area of RB1 and the mean % wall area of non RB1 airways generations 3-6 in the whole group ($r=0.69;p<0.0001$), pooled asthma cohort ($r=0.47;p=0.02$), EB ($r=0.78;p=0.008$) and healthy subjects ($r=0.81;p=0.0025$).(figure 3.28a)

Airway wall thickening and reduced luminal patency is a feature of asthma but not EB in non RB1 airways generations 3-6.

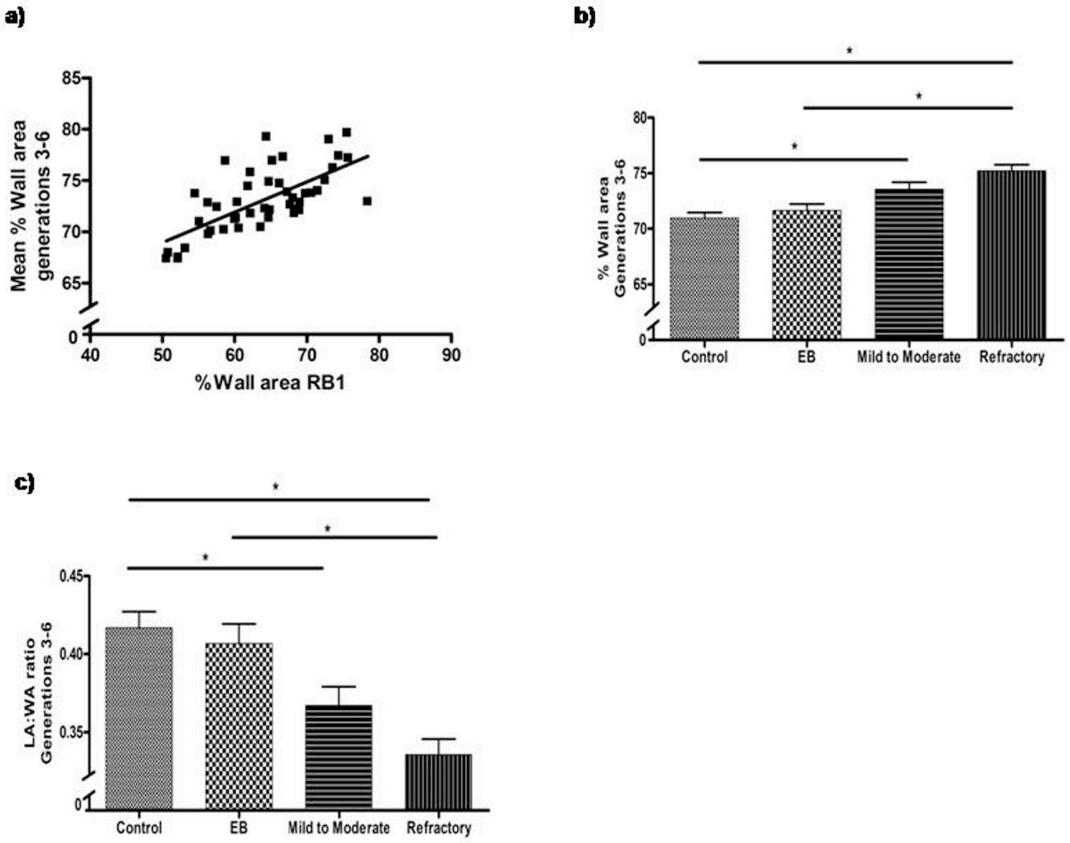
In non RB1 airways (generations 3-6) the mean (SEM) %WA was significantly increased in the subjects with refractory asthma (75.2 [0.54]%), and mild to moderate persistent asthma (73.6 [0.64]%) compared to the control group (71.0 [0.50]%). In contrast a significant difference was only observed between refractory asthma and the EB cohort (71.7 [0.58]%), $p<0.0001$, (Figure 3.28b). Conversely the mean (SEM) ratio of the BSA corrected LA:WA was significantly reduced in subjects with mild to moderate persistent asthma (0.37[(0.01]) and refractory asthma (0.34[0.01]) compared to healthy controls (0.42 [0.01]). In contrast a significant difference in LA:WA was only observed between refractory asthma and the EB cohort (0.41(0.01), $p<0.0001$, (Figure 3.28c).

Figure 3.28 Reduced patency and airway wall thickening in non RB1 airways generations 3-6 in asthma but not EB.

a: *Correlation of RB1 % wall area and non RB1 airways generations 3-6 in the entire cohort (asthma, EB and healthy subjects).*

b: *Corrected %WA (mean(sem)) of non RB1 airways [generations 3-6] in subjects with asthma, EB and healthy controls. *p<0.05 .*

c: *LA:WA ratio (mean(sem)) of non RB1 airways [generations 3-6] in subjects with asthma, EB and healthy controls. *p<0.05.*



Discussion

We have shown for the first time that subjects with EB have maintained patency of the right upper lobe apical bronchus (RB1) a third generation proximal airway and other conducting airways generations 3-6 without evidence of airway wall thickening. In contrast subjects with asthma had evidence of airway wall thickening and reduced luminal patency. There was a strong correlation between proximal airway wall thickening and AHR in asthma. EB is therefore characterised by maintained proximal airway patency whereas in contrast asthma is associated with proximal airway wall remodelling and luminal narrowing. The static geometry of the airway may protect against airway hyperresponsivenes in EB, but promote it in asthma.

We chose to use CT as a measure of airway remodeling as it provides a global measure of wall geometry that cannot be derived from endobronchial biopsies. Indeed CT has emerged as a useful tool to study airway wall remodeling in asthma and COPD[65;67-69;96]. We have demonstrated that geometric measurements are highly repeatable between observers and that correction equations can be derived from phantom studies to adjust for size dependant bias in airway geometry due to partial volume averaging as airways become smaller. Furthermore we have demonstrated that airway length can be accurately measured.

Using CT we have demonstrated that disordered airway function is related to the thickening of the proximal airway wall in asthma. This view is strengthened by the absence of airway wall thickening in EB, a condition that shares many of the immunopathological features of asthma without variable airflow obstruction or AHR[364]. In contrast EB was characterised by maintained airway patency of the proximal airway lumen. We therefore reject our original hypothesis. As airway hyper responsivenss is dependant upon proximal wall thickening in asthma and airway wall thickening is absent in EB in proximal airways. The preceding features of remodeling may contribute

to the absence of AHR in this disease control model. Interestingly, in bronchiectasis proximal dilatation is protective against airflow limitation[406]. Our findings extend the earlier observations by Park et al[318] who described the absence of proximal airway wall thickening in EB together with evidence of distal disease as evidenced by centrilobular opacification within secondary pulmonary lobules and air trapping in expiratory CT scans. The presence of distal airway disease may account for why some patients with EB develop fixed airflow obstruction[312]. However, we chose EB subjects with normal lung function with repeated measures over several years and therefore are likely to have excluded the subgroup of EB subjects that develop airflow obstruction.

In comparative studies of EB and asthma we have reported that increased vascularity, ASM mass and reticular basement membrane thickening are features of both diseases[314;368;380]. These earlier findings suggest that remodeling and AHR are dissociated. However, our current study provides evidence that AHR and altered airway geometry are correlated. One possible explanation for this apparent paradox is that remodeling occurs in both diseases but in asthma the remodeling process occurs towards the lumen leading to airway narrowing and wall thickening, whereas in EB the remodeling occurs away from the lumen leading to relative airway dilatation and an increase in the total area of the airway compared to the asthmatic subjects. An alternative or perhaps synergistic mechanism to explain the difference in airway function between asthma and EB is the difference in localisation of mast cells within the airway wall. We and others have demonstrated that mast cells are microlocalised within the airway smooth muscle bundle in asthma and that this is associated with AHR (reviewed in[106]) and the inability of some asthmatics to bronchodilate in response to a deep breath[324]. It is also important to note that comparative studies of airway structural changes in EB and asthma are incomplete and further work is required to assess other features of airway remodelling in EB such as matrix deposition in the bronchial lamina propria and airway smooth muscle mass.

We were unable to demonstrate any change in static geometry in mild to moderate asthma or EB after a 2-week trial of oral prednisolone despite improvement in markers of airway inflammation. In contrast Matsumoto *et al* have shown that treatment with inhaled budesonide led to a significant reduction in airway wall thickening of RB1 after 12 weeks in asthma without any further reduction in wall calibre after a median follow up of 4.2 years[407]. This would suggest that the anti-remodeling effects of corticosteroids may have a latency of between 2 and 12 weeks.

One limitation of our study is its cross-sectional design. We therefore do not have longitudinal data to support the notion that the increased luminal and total airway area in those subjects with EB compared to asthma truly represents remodeling rather than an innate geometric airway advantage. However, from longitudinal case series we recognise that some subjects with EB can develop fixed airflow obstruction supporting the view that remodeling does occur in EB[312]. Another potential shortcoming is that we did not use respiratory gating to gate CT scans to full inspiration. However, animal studies have shown that there are negligible effects of lung volume on static airway calibre at transpulmonary pressures >10 cmH_{2O}[408], suggesting that spirometric gating is unlikely to be necessary if a good respiratory effort is made. Furthermore we did not administer a short acting bronchodilators prior to CT scanning. However all patients with refractory asthma and 5/11 patients with mild to moderate persistent asthma took a long acting bronchodilator 2 hours prior to their CT scan which would have minimized the effect of baseline airway smooth muscle tone upon airway narrowing that has been demonstrated in mathematical models[20;288]. We did not demonstrate any difference in % wall area, luminal area or RB1 length in the mild to moderate persistent asthma cohort between patients that had taken a LABA prior to CT imaging and those that had not, furthermore we failed to observe any difference in length between asthma and EB suggesting that the observed differences in static geometry were not due to increased smooth muscle tone in asthma. Finally the definition of eosinophilic bronchits is exclusive of airflow limitation[364] and further studies are required to assess the geometry of proximal airways in patients with eosinophilic airway inflammation, fixed airflow obstruction with an absence of lower airway hyperresponsiveness.

In summary we have shown that airway wall geometry is differentially altered in asthma and EB. Maintained proximal airway patency in EB and increased total airway area compared to the subjects with asthma may protect against the development of AHR, whereas airway wall thickening and luminal narrowing may promote AHR in asthma.

3.2.3

Proximal airway remodeling in severe asthma: Relationship with airway inflammation.

Abstract

Background. CT of the right upper lobe apical segmental bronchus (RB1) is a useful measure of proximal airway wall remodelling in asthma. We sought to identify whether there are differences in the static geometry of the RB1 bronchus in patients with eosinophilic and non-eosinophilic inflammatory phenotypes of severe asthma.

Methods. The geometry of the RB1 bronchus was measured using the full width half at maximum (FWHM) method in 30 patients with severe eosinophilic asthma, 16 patients with non-eosinophilic asthma and 10 healthy controls that had undergone thoracic HRCT. CT scanning was performed at 120kVp, 140mAs, 16x1mm collimation. Two observers measured RB1 cross-sectional geometry.

Results. Severe asthma with or without eosinophilic airway inflammation was associated with significant thickening of the RB1 bronchus mean (sem) %wall area (eosinophilic asthma 70.5(1.2)% and non-eosinophilic asthma 70.6(1.3)% compared to the control group 63.1(1.4)%; p=0.004). The mean (sem) luminal area/body surface area mm^2/m^2 was reduced in subjects with eosinophilic asthma in the whole cohort (eosinophilic asthma 5.6(0.48)mm^2/m^2, non-eosinophilic asthma 6.0(0.6)mm^2/m^2 and control group 7.9(0.58)mm^2/m^2; p=0.03) and in those with non-eosinophilic asthma and disease duration of >20 years (5.28(0.95) mm^2/m^2; $p<0.05$).

Conclusions. Airway wall thickening of proximal airways is independent of eosinophilic airway inflammation in severe asthma.

Introduction

Asthma is a common disease that is rising in prevalence worldwide. About 5-10% of the asthma population have severe disease. This group is important as they are responsible for a disproportionate share of the health care costs and morbidity associated with this disease[330]. One of the cardinal features of the asthma phenotype is airway inflammation. The identification of eosinophilia using induced sputum[210] has emerged as a powerful tool in asthma to predict response to inhaled[221] and oral corticosteroids[53] and targeting eosinophilia has been repeatedly shown to reduce severe exacerbations[55-57]. However 50% of patients with treated asthma do not have a sputum eosinophilia[151] and non-eosinophilic asthma (NEA) has been shown to extend across the spectrum of severity[80;226;409] and seems to represent a stable phenotype of the asthma paradigm[55;56;228].

Remodeling of the airway wall characterised by structural changes such as increased smooth muscle mass, vascular remodeling and subepithelial fibrosis has been shown to be associated with airflow limitation[76;368]. Furthermore cellular inflammation of the airway wall in asthma is associated with an accelerated decline in FEV_1[350]. Few studies have characterised the histopathology of NEA. Two studies spanning the spectrum of asthma severity have previously demonstrated that thickening of the reticular basement membrane is absent in NEA in contrast to subjects with eosinophilic airway inflammation[80;161], suggesting that there may be architectural differences within the airway wall between these two asthma phenotypes.

Computed tomography has emerged as a repeatable and accurate tool to measure airway wall thickening of proximal airways in asthma[67;68]. Thickening of the RB1 apical bronchus of the right upper lobe has been shown to correlate with airflow limitation and airway hyperresponsiveness in asthma[67], as well as distal airway wall thickening in COPD[392]. Whether remodeling assessed by CT is a feature of eosinophilic asthma (EA) and NEA is unknown.

In the present study we have assessed the geometry of the RB1 bronchus in severe asthma in patients with and without eosinophilic airway inflammation to establish whether there are differences in proximal airway wall remodeling.

Materials and Methods

Subjects

We performed a cross sectional study based upon the difficult asthma clinic at Glenfield hospital, Leicester, UK in patients attending between 2/2000 and 11/2006. Subjects attending the difficult asthma clinic undergo an extensive re-evaluation, as part of their routine clinical care including an extensive history, skin prick tests for common aeroallergens, spirometry, methacholine challenge tests, sputum induction[210] and asthma control questionnaire[410]. The diagnosis of asthma is confirmed by a physician and one or more of the following objective criteria (peak flow amplitude % mean of >20% over a 2 week period, significant bronchodilator reversibility defined as an increased in FEV_1 of >200mls post bronchodilator or a PC_{20} methacholine of <8mg/ml). Only asthmatic subjects with <10 pack year smoking history were included. The non-asthmatic controls were non-smokers with normal spirometry. Informed consent for clinical characterisation and computed tomography was obtained from all subjects and the study was approved by the Leicestershire, Northamptonshire and Rutland Ethics Committee.

Protocol.

We defined NEA as those subjects with asthma that had a sputum eosinophil count of ≤1.9%[12] on at least 2 occasions with no previous evidence of significant eosinophilia >1.9% and EA as those subjects with asthma and a sputum eosinophil count of ≥3%[221] on at least 2 occasions with a maximal eosinophil count of >10%. We recruited subjects that had EA (n=30) or NEA (n=16) and a digitalised HRCT scan with a measurable RB1 bronchus (subject recruitment is summarised in figure 3.29). There were no significant differences in demographics between those subjects that did or did not have an assessable CT scan in subjects with EA or NEA.

Cross sectional imaging and airway analysis

HRCT scanning was performed using a Siemens Sensation 16 scanner at 120kVp, 140mAs, and 16x1mm collimation. All scans had been reported by an experienced radiologist prior to analysis. The RB1 bronchus was measured using the FWHM method using a custom program (Emphylyx-J V 1.00.01)[398]. Two observers blinded to the subjects' clinical data [SG, SS] performed airway measurements. Airway measurements were corrected for body surface area[405] as well as oblique orientation and size dependant error using equations derived from the phantom airway validation model [see previous CT validation section].

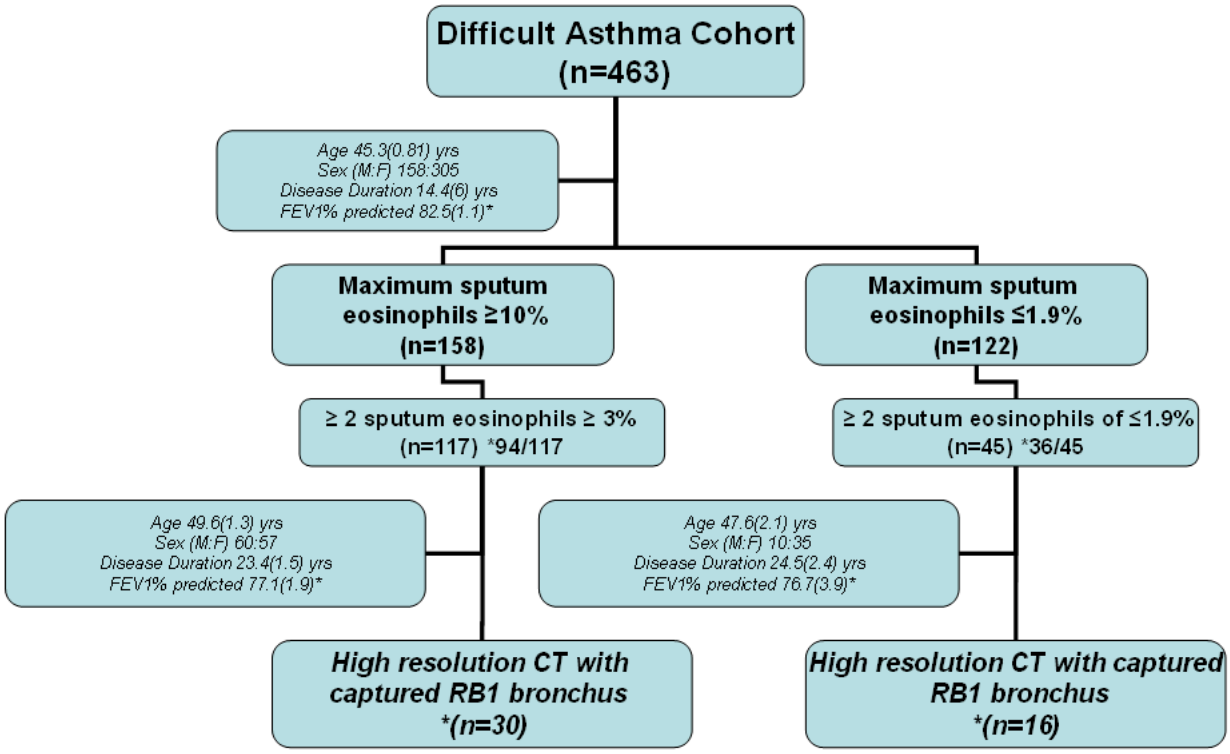
Table 3.16: Clinical Characteristics

	Control (n=10)	Eosinophilic Asthma (n=30)	Non-Eosinophilic asthma (n=16)
Age (yrs)	49.2(4.7)	49.9(2.4)	50.1(5.2)
Sex M:F	6:4	15:15	5:11
Disease Duration (yrs)	na	20.1(3.2)	31.4(5.6)
Atopy (%)	x	50	60
BMI (kg/m^2)	26.3(1.9)	28.5(0.8)	29.0(1.5)
Post BD FEV_1	3.6(0.4)	2.5(0.2)	2.3(0.3)*¶
Post BD FEV_1%	101.2(4.8)	81.4(3.5)	77.8(7.9)*¶
Post BD FEV_1/FVC	80.6(2.1)	71.7(1.8)	71.1(4.0)
Best FEV_1 BDR (%)	x	20.0(2.6)	21.3(5.2)
Inhaled BDP equivalent/24 hrs (mcg)	na	1907(162)	2115(246)
Oral Prednisolone (mg/24hrs)	na	6.2(1.2)	7.2(2.7)
Oral corticosteroids (%)	na	53	44
Inhaled SABD puffs/24hrs	na	3.4(0.5)	4.1(1.0)
Long acting bronchodilator (%)	na	90	94
Juniper asthma control score	na	2.0(0.2)	2.6(0.3)
Courses of oral prednisolone taken in the preceding year	na	2.0(0.4)	2.0(1.0)
Refractory asthma (ATS Consensus)**	na	27/30	13/16
Sputum eosinophils (%) #	x	5.6[3.2-9.6]	0.44[0.29-0.66]^^
Sputum neutrophils (%)	x	50.7(5.3)	64.6(6.2)

*Data expressed as mean (SEM); #Geometric mean [95%CI]. Beclamethasone Diproprionate (BDP) equivalents; fluticasone 2:1, Budesonide 1.25:1, Mometasone 1.25:1. Intergroup comparisons: t-test and one-way ANOVA with Bonferroni correction for multiple comparisons. ** Refractory asthma according to American Thoracic Society (ATS) consensus criteria[9]. BD= Bronchodilator, BDR= Bronchodilator response, SABD= Short Acting Bronchodilator.* $p<0.05$; One Way Analysis of Variance: ¶ $p<0.05$; NEA/EA vs. Control, ^^ $p<0.05$; NEA vs. EA.*

Figure 3.29: Patient selection diagram

*The Difficult asthma cohort (n=463) was screened for potential patients with non eosinophilic asthma (NEA) and eosinophilic asthma (EA). Patients with a stable phenotype and digitally archived CT scan subsequently has geometric measurements of the RB1 apical segmental bronchus (right upper lobe). *patients with a <10-pack year smoking history.*



Analysis.

Statistical analysis was performed using PRISM Version 4 (Prism, La Jolla, Calif) and linear regression using SPSS Version 13.0 (SPSS Inc, Chicago, III). Parametric data were expressed as mean (SEM), log normally distributed data was described as geometric mean (95% CI) and non-parametric data were described as median (range). Student's t-tests and Chi-squared tests were used for between group comparisons (two groups) and categorical data respectively.

One-way analysis of variance with Bonferronis correction (normally distributed data) and Dunns intergroup comparison (non-normally distributed data) was used to compare multiple groups; data are presented as the p value for the ANOVA model and intergroup comparisons were quoted as significant if the post test showed a p value of <0.05.

Stepwise linear regression was performed for %WA with age, daily equivalent dose of inhaled beclomethasone diproprionate and oral corticosteroid, disease duration, body mass index, sputum eosinophil count and neutrophil count and post bronchodilator FEV_1% predicted as the independent variables. A p value of <0.05 was taken as statistically significant.

Results

There was excellent agreement between observers for the measurement of % wall area (WA) of the RB1 bronchus r_s=0.8; p<0.0001 (n=55).

Thickening of the RB1 bronchus in severe asthma is independent of eosinophilic airway inflammation.

The mean (sem) %WA of the RB1 bronchus was significantly increased in both EA [70.5(1.2)%] and NEA [70.6(1.3)%] compared to the control group [63.1(1.4)%] (p=0.004; figures 3.30&3.31a).

Reduced luminal patency in severe asthma with eosinophilic airway inflammation.

The mean (sem) luminal area/ body surface area (LA/BSA mm^2/m^2) of the RB1 bronchus was significantly smaller in EA [5.6(0.48)] compared to the control group [7.9(0.58] p= (0.04); but not compared to NEA [6.0(0.6)]. (figure 3.31b)

Figure 3.30: CT images of the RB1 bronchus.

HRCT images at x5 magnification of the RB1 bronchus (red boxes) illustrating a) A control with an absence of airway wall thickening and preserved luminal calibre, b) Severe eosinophilic asthmatic with airway wall thickening and narrowing of the airway lumen and c) A severe asthmatic without eosinophilic airway inflammation with airway wall thickening and relative luminal narrowing.

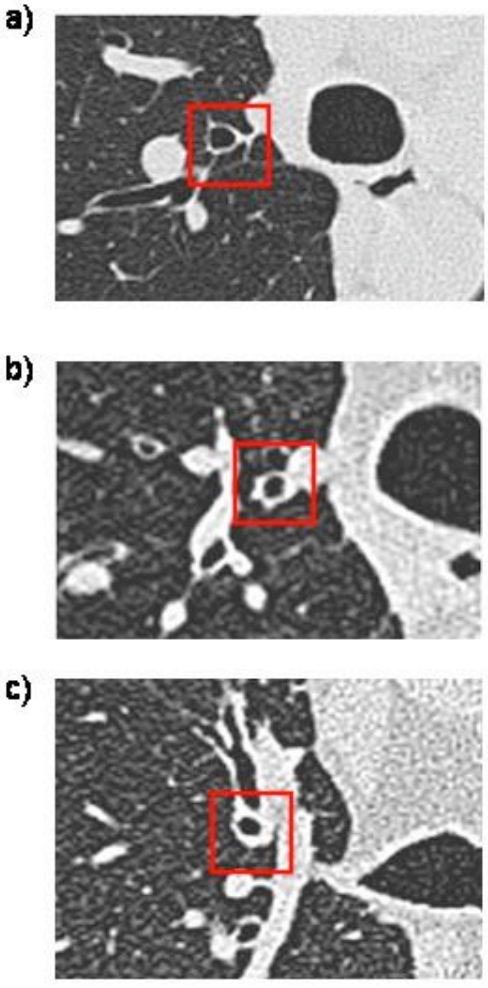
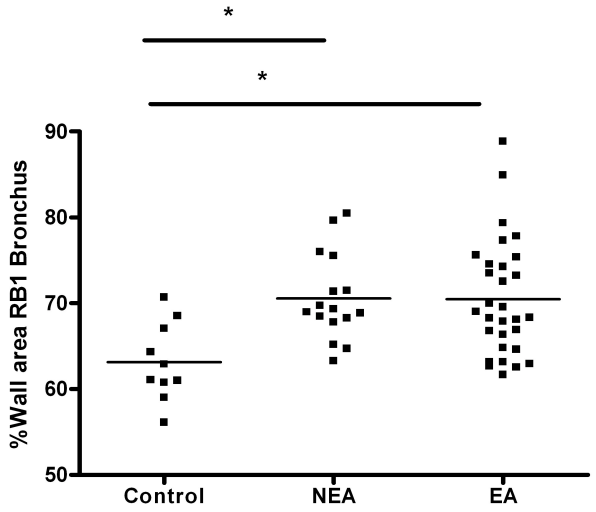


Figure 3.31: Increased %WA of RB1 in asthma independent of EAI.

*a) The % wall area of the RB1 apical segmental (right upper lobe) bronchus in eosinophilic asthma (EA), non eosinophilic asthma (NEA) and control subjects. * p<0.05. b) Reduced Luminal area of RB1 in eosinophilic asthma*

a)



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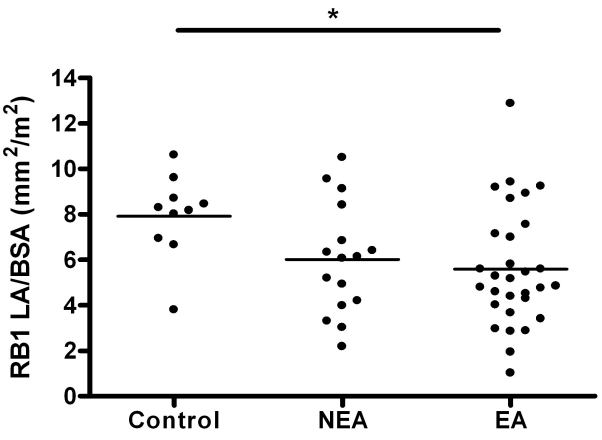


Thickening of the RB1 bronchus in severe asthma is associated with airflow limitation.

Univariate analysis demonstrated that the %WA of RB1 correlated with the FEV_1% predicted (r_s = -0.31; p=0.035), age (r_s = 0.31; p=0.029) and disease duration (r_s =0.43; p=0.003) in the pooled asthma cohort.

Step-wise linear regression demonstrated that disease duration best modelled the %WA of RB1 in the pooled severe asthma cohort (Model adjusted R^2=0.09, β=0.33; p=0.016).

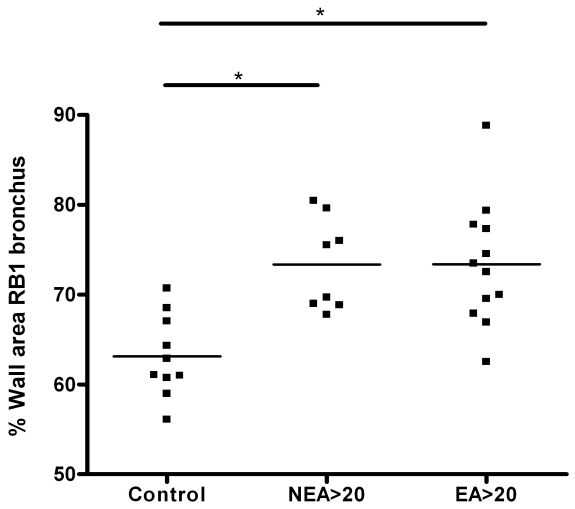
Reduced luminal patency in severe asthmatics with chronic disease is independent of eosinophilic airway inflammation.

Based upon the regression results, each group was stratified according to disease duration of </= or > 20 years. The LA/BSA and %WA of the RB1 bronchus were significantly smaller and greater respectively in patients with disease duration of >20years; NEA [5.28(0.95) mm^2/m^2; 73.35(1.8)% (n=8)] and EA [4.65(0.58) mm^2/m^2; 73.4(2.0)% (n=12)] compared to the control group [6.0(0.6) mm^2/m^2; 63.1(1.4%)] p<0.05 (figure 3.32).

Figure 3.32: Reduced luminal patency of the RBl bronchus in asthma is independent of eosinophilic airway inflammation in patients with chronic disease.

a) % Wall area and b) luminal area (mm^2/m^2) of the RB1 bronchus in patients with eosinophilic asthma, non eosinophilic asthma and disease duration of > 20 years compared to control subjects. $p<0.05$*

a)



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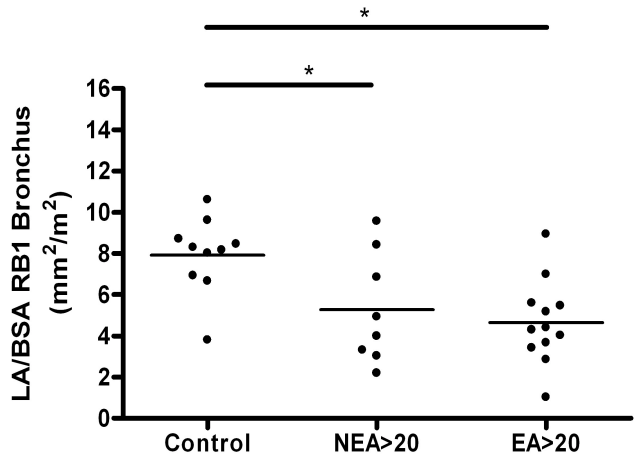


Discussion

We have shown for the first time that remodeling of the RB1 bronchus, a proximal third generation airway is independent of eosinophilic airway inflammation. We found that reduced patency of the airway lumen was present in severe asthma with eosinophilic airway inflammation and in non eosinophilic asthma in the presence of chronic disease. We have also demonstrated that the thickness of the RB1 bronchus is associated with airflow limitation and duration of disease in severe asthma.

The identification of eosinophilic airway inflammation in severe asthma has evolved as a powerful diagnostic tool to predict response to corticosteroids[53] and to prevent exacerbations[55;56]. The sensitivity of induced sputum as a proxy for granulocytic inflammation in the airway wall is relatively poor; however the absence of eosinophilic airway inflammation in sputum has a high negative predictive value for the absence of eosinophils within the airway wall in severe asthma[411]. Furthermore eosinophilic inflammation in the airway wall failed to identify patients who subsequently exacerbated compared to induced sputum in one study[412]. Therefore we are confident that the use of induced sputum to quantify granulocytic airway inflammation in the present study was valid.

Our study is the first study to examine static airway geometry in severe asthma stratified according to airway inflammation in patients with a stable inflammatory phenotype.

Our observation of airway wall thickening in severe asthma being independent of EAI is in keeping with other imaging studies. Little et al failed to find an association between airway wall geometry and sputum neutrophils or eNO[413]. Similarly Niimi et al failed to find an association between sputum eosinophilia or serum ECP and airway wall geometry in two quantitative airway imaging studies[67;68]. Paradoxically low serum eosinophil cationic protein (ECP) levels were associated with a greater degree of wall thickening on CT than those patients that had high ECP levels during exacerbations in one study[414].

In contrast De Blic et al demonstrated in a childhood asthma cohort that broncho alveolar lavage ECP levels were related to airway wall thickening in asthma[415]. However no correlation was found between intraepithelial neutrophils or eosinophils and airway wall thickness in keeping with the present study. Similarly in a separate study Niimi et al demonstrated using a quantitative airway algorithm that the change in airway wall dimensions on CT after 12 weeks of BDP 800mcg daily was related to the change in serum ECP[66]. Furthermore other biomarkers of inflammation in sputum such as TGF-β[416] and the ratio of MM9 to TIMP-1[63] have been associated with airway wall thickening in imaging studies

The discordancy between these imaging studies suggests that airway geometry is not a simple product of granulocytic airway inflammation and may be related to eosinophil activation rather than the number of eosinophils within the airway wall or the flux of these cells into to surface lining fluid as well as other factors such as duration of disease, asthma control, asthma severity and treatment

Pathological studies of the airway wall in asthma suggest that eosinophilic inflammation has an important impact upon structural airway remodeling. Comparisons between asthma and non-asthmatic eosinophilic bronchitis have revealed that reticular basement membrane thickening is related to the presence of eosinophilic airway inflammation rather than asthma per se[105;314]. Few pathological studies have compared the immunopathology of asthma with or without eosinophilic airway inflammation. One study in mild steroid naïve asthmatics found that reticular basement membrane thickening was present in asthma with eosinophilic airway inflammation only and that mast cell smooth muscle myositis was present in asthma independent of eosinophilic airway inflammation. Another study in severe asthma found that patients with significant eosinophilic airway inflammation of the airway wall had a thicker reticular basement membrane than those without eosinophilic inflammation and the degree of submuscosal eosinophilia correlated with the degree of basement membrane thickening[80]. These findings are in keeping with animal models in eosinophil depleted mice, that have shown that thickening of the RBM is related to eosinophilic airway inflammation[162]. However no studies have assessed whether there are differences in

other airway structural elements such as airway smooth muscle, matrix and glands in patients with asthma with or without eosinophilic airway inflammation.

We demonstrated a reduction in the patency of the airway lumen in severe asthmatics. This finding is in keeping with a recent study in severe asthma that quantitatively assessed remodelling in 19 bronchial proximal airway segments post bronchodilatation and demonstrated that the % area of the airway lumen was significantly smaller in severe asthma compared to healthy subjects and patients with mild to moderate persistent asthma. Furthermore this study found a close association between wall thickening of the RB1 bronchus and the mean wall thickness in non RB1 proximal airways[417]. The majority of our patients with severe asthma had long acting bronchodilators (15/16 NEA and 27/30 EA) prior to the CT scan and we are confident that this would have minimised the confounding effect of increased ASM tone on airway luminal geometry.

Our finding of thickening of the airway wall independent of eosinophilic airway inflammation would suggest that the presence of the eosinophil is not critical for remodeling. It therefore raises the question of which pathological features of remodeling, such as increased airway smooth muscle mass, vascular remodeling, glandular hypertrophy and increased matrix, are associated with NEA. We have not compared the changes in the airway wall between the proposed subtypes of NEA i.e. neutrophilic and paucigranulocytic asthma. However, we did not find an association between the degree of sputum neutrophilia and the degree of remodeling of the RB1 bronchus. Further studies are required to assess the impact of neutrophilic airway inflammation with or without sputum eosinophilia upon airway wall remodelling in severe asthma. Interestingly, reduced patency of the airway lumen irrespective of disease duration was only observed in severe asthma with eosinophilic airway inflammation, which may reflect thickening of the inner airway wall due to reticular basement membrane thickening. However this needs to be confirmed by other studies analysing static geometry and histopathology of the airway wall.

We have demonstrated that disease duration was independently associated with thickening of the airway wall in severe asthma in keeping with previous reports that have shown that vascular remodeling of the airway wall is related to disease duration[368] and that duration of disease was associated with increased airway smooth muscle mass and luminal narrowing in patients with fatal asthma[418]. We have also demonstrated that thickening of the RB1 bronchus is associated with airflow limitation in severe asthma; in keeping with a number of studies that have linked the static geometry of this airway to airflow limitation in both asthma and COPD[68;392].

Our study has a number of limitations. The cross-sectional design does not allow us to explore the relationship between disease progression and CT evidence of remodeling over time and longitudinal studies would be informative. The HRCT scan was part of the clinical assessment of the subjects and was not undertaken in all subjects. In addition, we analysed images of the RB1 bronchus from standard HRCTs and not limited CT scans, which capture the RB1 bronchus across its entire length. Therefore there is the potential for bias in our group of patients with EA and NEA. However, we are confident that our strict definition of EA and NEA enabled us to compare these inflammatory phenotypes. Importantly, we did not identify any differences in the baseline demographics between the whole population of EA or NEA and the subgroup with assessable HRCT scans. We are also unable to comment on the relationship between pathological features of remodeling and CT changes as we do not have matched CT and bronchial biopsies. Future studies that include both modalities to assess remodeling are required. Finally all of the asthmatics in this study were treated with inhaled and many with oral corticosteroids therefore we cannot exclude the possibility that eosinophilic inflammation may have been masked by treatment in some of the NEA subjects.

In conclusion we have shown for the first time that airway wall thickening and narrowing of the airway lumen in severe asthma are independent of eosinophilic airway inflammation and associated with airflow limitation and duration of disease. Longitudinal studies are required to assess the impact of highly selective anti-granulocytic agents upon airway remodeling in severe asthma.

4 Conclusions

4.1 Summary of findings

This thesis has examined for the first time features of structural remodeling in asthma and EB and airway geometry in these two polar conditions.

We have demonstrated for the first time that structural remodeling of the airway wall; notably increased ASM mass, thickening of the reticular basement membrane and collagen 3 deposition, vessels and glands within the lamina propria are dissociated from airway hyper responsiveness in asthma and are present in patients with non asthmatic eosinophilic bronchitis (EB). These findings challenge the widely held conception that structural remodeling of the airway wall alone can mediate AHR in adult asthma.

In contrast we have confirmed and extended our previously reported finding of mast cell infiltration of the airway smooth muscle in asthma and the number of mast cells in the ASM bundle was increased in asthmatic compared to patients with EB and healthy subjects. In addition we found that mast cell infiltration of the ASM was an independent predictor of the degree of AHR in asthma.

Coupled with these findings we have shown for the first time that EB is characterised by maintained patency of the proximal airway lumen despite thickening of the airway wall, in contrast in asthma thickening of the airway wall led to a reduction in airway patency and was associated with AHR. Furthermore we have demonstrated that in severe asthma the degree of proximal airway wall thickening is independent of eosinophilic airway inflammation.

Finally we have shown for the first time that fibrocytes are present *in vivo* in the ASM and lamina propria in asthma compared to patients with EB and healthy subjects, suggesting that recruitment of smooth muscle progenitor cells to the airway wall may be important in mediating ASM hyperplasia seen in asthma. One could therefore speculate that different mechanisms drive the increased ASM mass seen in asthma and EB.

Alternatively fibrocytes may differentiate in to mast cells in the ASM bundle in asthma explaining their absence in EB and healthy subjects. In contrast to mast cells, fibrocyte numbers in the ASM did not correlate with AHR or airflow limitation in asthma.

Our findings suggest that the impact of remodeling upon airway patency and infiltration of the ASM bundle with mast cells are key co factors that regulate AHR in adult asthma. (Figure 3.33)

4.2 Techniques developed and problems encountered

A number of techniques were developed during the course of this thesis. In the laboratory we validated the measurement of VEGF in induced sputum supernatant and the chalkley count in tissue as a simple tool to quantify the degree of vascularity within the airway wall. We also developed and validated a method of thresholding matrix within the airway wall that correlated closely with the visual analogue of matrix intensity. Finally we validated the use of the full width at half maximum technique to measure airway wall geometry of the RB1 bronchus and determined the accuracy, repeatability and limit of resolution of the airway analysis algorithm.

One of the key problems encountered was the need to acquire CT images after a full breath hold in patients with asthma and we were unable to develop a protocol to spirometrically gate our CT imaging to the respiratory cycle due to a lack of integrated software that linked our CT scanner to a spirometric device and spirometer linked patient visors that instructed them when to hold their breath during CT scanning. However we ensured that our patients were practiced at taking breath holds to total lung capacity prior to their CT scan. A further problem was the need to correct for size dependant error in airway geometry that occurred using the full width at half maximum technique. We derived correct equations using a polystyrene phantom modelling the RB1 bronchus and smaller airways and demonstrated that the correction equations reduced error in a separate sheep *ex vivo* airway model.

4.3 Future studies

Future studies should further attempt to characterise ASM mechanics in eosinophilic bronchitis. In particular the degree of ASM contractility and signalling of calcium is unknown in EB at present. Furthermore there are no published studies of the response to deep breaths in EB and one would speculate that patients with EB would behave in a similar fashion to healthy subjects after deep inspiration ie: reduced airway resistance and bronchodilatation. Furthermore the immunopathological data in this thesis have focused on proximal conducting airways and little is known about small airway pathology in EB. Therefore future studies should investigate and compare small airway remodeling using transbronchial biopsy, expiratory CT imaging, nitrogen washout and markers of small airway resistance in asthma and EB.

The mechanisms by which mast cells promote AHR in asthma remain unclear. Future work should assess the impact of mast cells upon ASM contractile protein *ex vivo* expression and actin expression *in vivo*. Recent work by our group supports the notion that mast cells increase ASM actin expression both *in vitro* and *in vivo*[333], thus making the ASM more muscular. Furthermore the mechanisms that promote recruitment and retention of fibrocytes to the ASM in asthma and the interplay between these cells and mast cells will be an important area of future study.

Finally a key question is, how does the remodeling process in EB allow maintained proximal airway patency in contrast to asthma where the same degree of structural remodeling leads to a reduction in airway patency? Future work will need to assess the impact of different components of remodeling upon the micromechanical properties of the airway wall and there is a pressing need to develop tools more sensitive than spirometry to measure mechanical properties such as hysteresis and airway distensibility *in vivo*.

Table 3.17: Summary of the immunopathological differences between asthma and EB.

	Control	Asthma	EB
Reticular basement membrane thickening	**x**	++	++
Vessels and VEGF	**x**	++	++
ASM mass	**x**	++	++
Matrix	**x**	+/-	+/-
Glands	**x**	+/-	+/-
Fibrocytes in the airway smooth muscle	**x**	++	--
Mast cells in the airway smooth muscle	**x**	++	--
IL-13 Blood/Sputum/ASM	**x**	++	--
Proximal Airway wall thickening (% Wall area)	**x**	++	--
Proximal luminal patency	**x**	**Decreased**	**Maintained**

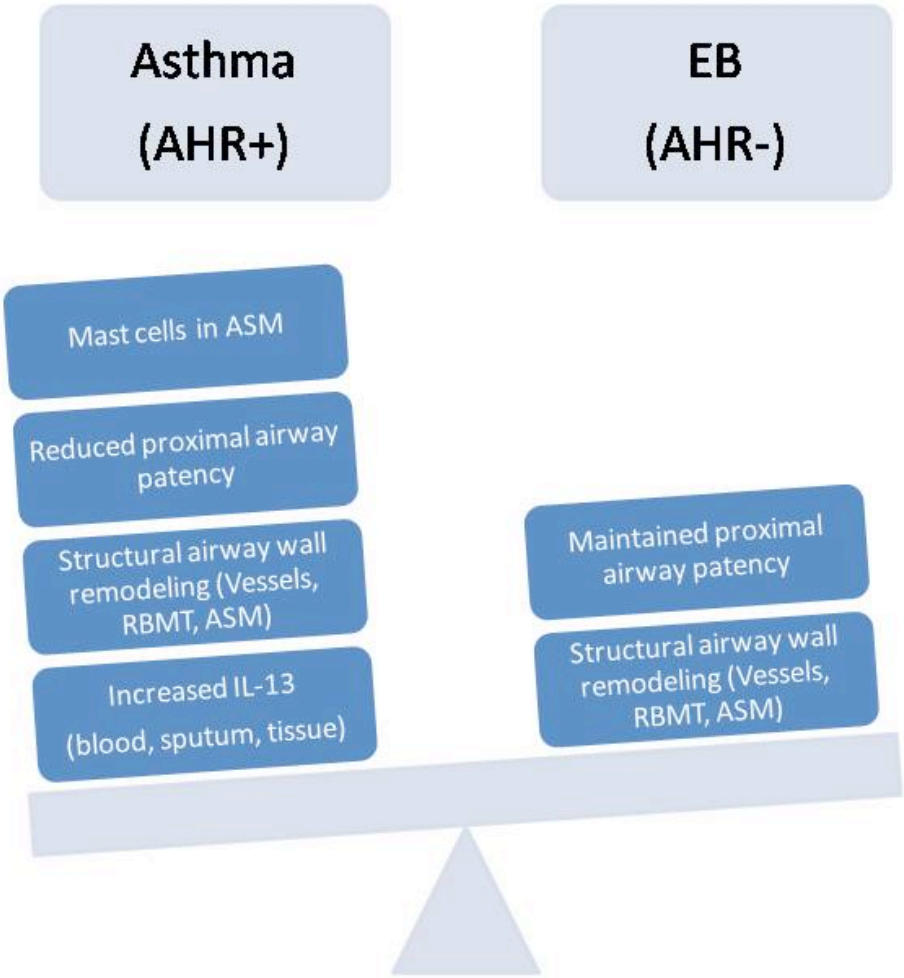
++ Significantly increased compared to healthy subjects

+/- Increased but not significantly compared to healthy subjects

- - Not increased compared to healthy subjects

x Not applicable (denominator group)

Figure 3.33: Mechanisms of AHR in adult asthma



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