AIRWAY STRUCTURAL REMODELLING IN ASTHMA: FUNCTIONAL RELEVANCE AND SUITABILITY AS A TARGET FOR THERAPY

Thesis submitted for the degree of Doctor of Philosophy (Ph.D.)

University of Leicester

By

Rachid Berair MB.BS, MRCP (UK), MRCP (Respiratory Medicine)

Department of Infection, Immunity and Inflammation

University of Leicester

February 2018

ABSTRACT

Airway structural remodelling in asthma: Functional relevance and suitability as a target for therapy

Dr Rachid Berair

Airway remodelling (AR) in asthma, a collective term describing all the airway microscopic structural changes, has long been known; however, its functional relevance is poorly understood. The lack of non-invasive methods for assessing AR contributes to this scarcity of studies on AR. First, this thesis describes the association of AR with physiological markers of airflow obstruction. This was coupled with attempting to assess the link between proximal and small airway qualitative computed tomography (QCT)-derived markers and AR. Furthermore, to further study the relevance of AR, we describe the effects of fevipiprant, a novel prostaglandin D2 (PGD_2) receptor 2 (DP_2) antagonist, and bronchial thermoplasty (BT) on various asthma domains including AR. We found that airway smooth muscle (ASM) mass and airway vascularity was closely related to airflow obstruction. Additionally, we have demonstrated that ASM, vascularity and epithelial thickness was associated with QCT-measured proximal airway morphometry changes whereas increased vascularity and goblet cells hyperplasia was related to air trapping. Coupled with improvements is eosinophilic inflammation, asthma symptoms and lung function, we have shown that DP_2 antagonism in a randomised controlled trial, resulted in improvement in epithelial integrity and reduction of ASM. Finally, we have shown that while BT treatment did not affect ASM mass, subepithelial fibrosis or lung function, it did improve epithelial integrity and reduced smooth muscle actin expression. Whether these changes contribute to the benefits seen in BT studies needs further research. This thesis has contributed to the development of fevipiprant as a new treatment for asthma, validated methods to assess AR, demonstrated how AR relates to asthma outcomes and shown how fevipiprant and BT impact AR. Further longitudinal studies are also needed to explore the heterogeneity of AR in various asthma phenotypes especially in the context of clinical trials of new therapies using novel non-invasive methods of measuring AR.

ACKNOWLEDGMENTS

Working as a clinical research fellow and undertaking this PhD at Glenfiled Hospital / University of Leicester has been truly life changing. None of it would have been possible without the support, guidance and help of many people.

First, I would like to thank my supervisor Professor Chris Brightling, without him I would not be where I am today. His enthusiasm and dedication to high quality clinical research is infectious. I would like to thank him for giving me the opportunity of undertaking this PhD in Leicester and more importantly for believing in me. He has always been a constant source of tremendous support, guidance and inspiration.

I am eternally grateful to all the patients and volunteers who contributed to the clinical studies and trials included in this thesis.

I would like to thank members of my progression review panel, Professor Salman Siddiqui and Dr Yassine Amrani for their support especially during the initial stages of my research. My special thanks to Amisha Singapuri and Beverley Hargadon for always being there for support and help throughout my time at Glenfield.

I would like to thank my fellow clinical research fellows. I would like to express my special gratitude to Sherif Gonem and Ruth Hartley for their help with various aspects of the studies included in this PhD.

I am grateful to Novartis Pharmaceuticals for providing funding and support for the Fevipiprant study. My thanks in particular are to Dr Richard Kay who was closely involved with all the stages of the clinical trial. I would also like to acknowledge AIRPROM for funding some of my work.

Many thanks to Vijay Mistry for his endless support with the immunopathology work. I would like to express a special gratitude to Nisha Rana and Mitesh Pancholi for their technical support with the sputum and biopsy work. I would like to thank Aarti Shokirota who was always happy to share her knowledge and experience especially with microscopy.

On a personal note, I would like to thank my parents and my brothers. None of what I am would be possible without you.

Finally, I would like to dedicate this work to my wife Tamara and my two boys Ali and Karim. Tamara, you have always been there for me. I am so grateful for your unconditional love and support. Truly without you none of everything good in my life, including this PhD, would have been.

STATEMENT OF WORK PERSONALLY PERFORMED

- **Associations in asthma between physiological markers of air flow obstruction, quantitative computed tomography and bronchial biopsy-derived airway remodelling:** I was involved in all the stages of this study. I participated in the design and conception of the study. I planned the data collection, analysed the results and co-wrote the published manuscript. I performed all the biopsy microscopic analysis and participated in the QCT analysis.
- **The effects of prostaglandin D2 receptor 2 antagonism on airway inflammation, asthma symptoms, lung function and airway remodelling in uncontrolled moderate-severe eosinophilic asthma:** I was one of two sub-investigators responsible for the day to day running of the trial. I assisted in patient recruitment and consenting. I performed sputum induction, spirometry and methacholine bronchial challenge testing. I performed all the biopsy microscopy work and assisted in most bronchoscopies. I authored the trial statistical analysis plan and co-wrote the published manuscript. I participated in results analysis and presented the results of the trial in the two major respiratory international conferences, the European Respiratory Society and the American Thoracic Society meetings.
- **An observational multicentre study examining airway remodelling and repair in patients with severe persistent asthma treated with bronchial thermoplasty, an imaging and immunopathological study:** I participated in the design and conception of this study and was the study coordinator. I authored the study protocol and personally obtained ethical approval. I initiated all the sites involved and I designed the electronic study database. I analysed all the biopsies and CTs and was involved in the day to day running of the study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PUBLICATIONS ARISING FROM THIS THESIS

Primary original research

- **Berair R***, Hartley R*, Mistry V, Sheshadri A, Gupta S, Singapuri A, *et al*. Associations in asthma between quantitative computed tomography and bronchial biopsy-derived airway remodelling. ***Eur Respir J.*** 2017 May 1;49(5). *Joint first authors.

- Gonem S*, **Berair R***, Singapuri A, Hartley R, Laurencin MF, Bacher G, *et al*. Fevipiprant, a prostaglandin D_2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial. ***Lancet Respir Med***. 2016 Sep;4(9):699-707. *Equal contribution.

- Kaur D, Gomez E, Doe C, **Berair R**, *et al*. IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk. ***Allergy*** 2015 May;70(5):556-67.

- Singh SR, Sullo N, Matteis M, Spaziano G, McDonald J, Saunders R, Woodman L, Urbanek K, De Angelis A, De Palma R, **Berair R**, *et al*. Nociceptin/orphanin FQ (N/OFQ) modulates immunopathology and airway hyperresponsiveness representing a novel target for the treatment of asthma. ***Br J Pharmacol***. 2016 Apr;173(8):1286-301.

- Chernyavsky I, Russell RJ, Saunders RM, Morris GE, **Berair R**, et al. *In vitro*, *in silico* and *in vivo* study challenges the impact of bronchial thermoplasty on acute airway smooth muscle mass loss. Manuscript submitted to *Thorax May 2017*.

Book chapter

- Haldar P, **Berair R**. Endotypes and Asthma. Book chapter in: Bernstein JA**,** Levy ML. **Clinical Asthma: Theory and Practice**. 1st edition. CRC Press, Taylor & Francis publishing, New York. Jan 2014. 329 pages.

Review articles (peer reviewed)

- **Berair R**, Brightling CE. Asthma therapy and its effect on airway remodelling. **Drugs**. 2014 Aug;74(12):1345-69.

- Hartley R, **Berair R**, Brightling CE. Severe asthma: novel advances in the pathogenesis and therapy. ***Pol Arch Med Wewn***. 2014;124(5):247-54.

- **Berair R**, Pavord ID. Rationale and clinical results of inhibiting interleukin-5 for the treatment of severe asthma. ***Curr Allergy Asthma Rep***. 2013 Oct;13(5):469-76.

- **Berair R**, Saunders R, Brightling CE. Origins of increased airway smooth muscle mass in asthma. ***BMC Med***. 2013 Jun 6;11:145.

- **Berair R**, Hollins F, Brightling CE. Airway smooth muscle hypercontractility in asthma. ***J Allergy***. 2013; 2013:185971.

Abstracts

- R Saunders*, H Kaul*, **R Berair***, A Singapur, *et al.* Fevipiprant (FEVIPIPRANT) reduces airway smooth muscle mass in asthma via antagonism of the prostaglandin D2 receptor 2 (DP2). ***Am J Resp Crit Care Med.*** 2017;195:A4677. *equal contribution.

- R Saunders, M Biddle, A Wright,L Chachi, A Sutcliffe, **R Berair**, C Brightling . DP2/CRTh2 is expressed by ASM cells in asthma and its inhibition suppresses ASM migration. ***Eur Respir J*** 2016, vol. 48. Suppl 60, PA4653.

- **R. Berair,** S. Gonem, *et al.,* **Late-Breaking Abstract:** Effect of FEVIPIPRANT, an oral prostaglandin D2 receptor (DP2/CRTh2) antagonist, upon bronchial epithelial integrity in treatment-resistant asthma in a randomized, placebo controlled study. ***Eur Respir J*** September 1, 2015, vol. 46. Suppl 59, OA290.

- **R. Berair,** S. Gonem, *et al.,* **Late-Breaking Abstract:** Effect of FEVIPIPRANT, an Oral Prostaglandin D2 Receptor (DP2/CRTh2) Antagonist, Upon Sputum and

Bronchial Eosinophilic Inflammation and Clinical Outcomes in Treatment-Resistant Asthma: A Phase 2a Randomized Placebo-Controlled Trial. ***Am J Resp Crit Care Med*** 191;2015:A6361.

- DJE Goold, V Mistry, A Singapuri, M Cooke, **R Berair**, CE Brightling; Sputum And Bronchial Biopsy Expression Of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodg) In Asthma Is Related To Neutrophilic Inflammation And Poor Asthma Control. ***Thorax*** 2014;69:A71.

- **R Berair**, S Gonem, *at al*. Association between airway inflammation, airway remodelling and physiological changes in asthma. ***Eur Respir J*** September 1, 2014, vol. 44 no. Suppl 58, P3865.

- **R. Berair**, V. Mistry, at al. Association between body mass index (BMI) and markers of eosinophilic inflammation in asthma. ***Eur Respir J*** September 1, 2014, vol. 44 no. Suppl 58, P3870.

- R Singh, N Sullo, M Matteis, G Spaziano, J McDonald, R Saunders, L Woodman, K Urbanek, A DeAngelis, R DePalma, **R Berair**, *at al.* Activation of nociceptin orphanin FQ (N/OFQ) – N/OFQ peptide (NOP) receptor system plays a key immunomodulatory role in asthma. ***Thorax*** 2013;68:A12-A13.

- S Gonem, **R Berair**, *et al*.; **Late-breaking abstract:** Phase 2a randomized placebo-controlled trial of the oral prostaglandin D2 receptor (DP2/ CRTh2) antagonist FEVIPIPRANT in eosinophilic asthma. ***Eur Respir J*** September 1, 2014, vol. 44 no. Suppl 58 2908.

- R Russell, A Singapuri**, R Berair**, *et al***.** Clinical and histological effects of Bronchial Thermoplasty in severe asthma. ***Eur Respir J*** September 1, 2017, vol. 50 no. Suppl 61, PA3031.

ABBREVIATIONS

ACQ	Asthma Control Questionnaire
AHR	Airway hyper-responsiveness
AQLQ	Asthma Quality of Life Questionnaire
AR	Airway remodelling
ASM	Airway smooth muscle
α-SMA	*Alpha*-smooth muscle actin
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BDP	Beclomethasone dipropionate
BT	Bronchial thermoplasty
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
$CRTH_2$	Chemokine receptor homologous molecule expressed on Th2 lymphocytes
CT	Computed tomography
DTT	Dithiothreitol
EBB	Endobronchial biopsies
ECM	Extracellular matrix
ECP	Eosinophilic cationic protein
ED	Emergency department
EMT	Epithelial-mesenchymal transition

ERS	European Respiratory Society
FeNO	Fraction of exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
FRC	Functional residual capacity
FVC	Forced vital capacity
GCs	Glucocorticoids
GILZ	Glucocorticoid-induced leucine zipper
GINA	Global Initiative for Asthma
GMA	Glycol methacrylate
H&E	Haematoxylin and Eosin
HMG-CoA	Hydroxy methyl glutaryl coenzyme-A
ICC	Intraclass Correlation Coefficient
IQR	Inter-quartile range
KCO	Transfer coefficient
LA	Lumen area
LABA	Long acting β2-adrenergic receptor agonists
MLD E/I	Mean lung density expiratory/inspiratory
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
OCT	Optical coherence tomography

PGD_2	Prostaglandin D2
PMSF	Phenylmethane sulfonyl fluoride
QCT	Quantitative computed tomography
RBM	Reticular basement membrane
RCT	Randomised controlled trial
RV	Residual volume
TA	Total area
TGF-β	Transforming growth factor *beta*
TIMPs	Tissue inhibitors of metalloproteinases
TLC	Total lung capacity
TLCO	Carbon monoxide transfer factor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
WA	Wall area
WA%	Wall area percentage
WT	Wall thickness

1. CHAPTER ONE: INTRODUCTION AND BACKGROUND

1.1. History, definitions and epidemiology of asthma

1.1.1. History of asthma

The term "asthma" originates from the Greek word for panting "*aazein*" a verb meaning to exhale with open mouth. Although the word appeared in the Iliad, the first medical text where the word was coined as a medical term was in the Corpus Hippocraticum, a collection of texts and works associated with the ancient Greek physician Hippocrates (460-360 BC)[1]. He described it as episodic attacks of severe shortness of breath and recognised that these tended to occur more commonly among fishermen and tailors. However, Hippocrates probably was only describing a collection of symptoms rather than the pathological disease entity we know today [1]. Symptoms and signs of asthma have been described long before that by the ancient Egyptians and Chinese. Interestingly, the ancient Chinese even suggested the plant *Ephedra,* from which the bronchodilator ephedrine is derived, for the treatment of asthma-like symptoms.

In the 19th century, Henry Hyde Salter, an English physician, in his book titled "On Asthma and its Treatment", was the first to describe asthma as a distinctive disease and suggest that the paroxysmal nature of the illness was due to the spasms of the bronchial airways [2]. Remarkably, he also suggested coffee, a xanthine derivative similar to theophylline with bronchodilator properties, as a possible treatment for asthma [3]. The evidence for the significance of bronchial airways spasms was first demonstrated by Huber and Koesser in 1922 when they reported increased airway smooth muscle (ASM) mass in a small group of patients who died of status asthmaticus compared to patients who died from non-pulmonary conditions [4]. Furthermore, multiple bronchodilators were discovered and shown to be effective in the treatment of asthma attacks, thus further reinforcing the idea that asthma is purely an ASM abnormality.

Since the discovery of eosinophils and mast cells stains (eosin and toluidine blue respectively) by Paul Ehrlich in the late 19th century, inflammation has been recognized to occur in the asthmatic airways. However, the relevance of inflammation was not suspected until the observation of asthma death epidemics associated with overuse of bronchodilators. Although oral corticosteroids have been used in the treatment of asthma since the 1950s, the identification of asthma as an inflammatory disease had to wait until the 1980s when the importance of IgE medicated allergy and Th_2 immunity in the pathogenesis of the disease was better defined.

1.1.2. Definition of asthma

As a pathological disease entity, there is no gold standard universally agreed definition of bronchial asthma. The definition has evolved and changed over time and was always reached by consensus agreement and based mainly on the observable clinical and physiological characteristics seen in patients.

The most recent report issued by the Global Initiative for Asthma (GINA) states that asthma is defined by episodic symptoms of cough, breathlessness, chest tightness and wheeze together with variable airflow obstruction. It recognises that airway inflammation, remodelling and hyperresponsivness are frequently present; however they are not essential or enough to define or diagnose the disease [5]. This is also shared by the definition stated in the recent British guidelines [6]. This clearly reflects the lack of understanding of various aspects of the disease especially at the microscopic tissue level.

1.1.3. Asthma epidemiology and economic burden

In 2004 it was estimated that asthma affected 300 million globally, a number expected to increase to 400 million by 2025 [7]. The prevalence of asthma is 5-10% and it remains the most common chronic condition affecting children. It is associated with significant

morbidity and mortality especially in patients with the severe form of the disease who account for 5-10% of all patients in both the adult and paediatric populations. Globally asthma is responsible for 250000 deaths annually. Although most of these deaths are in low and middle income countries, in developed counties asthma still results in significant mortality.

In the UK, asthma carries a significant health and economic burden. It affects 5.4 m people, 10% of which have severe disease. Mortality from asthma in the UK remains high with an annual average of 1200 deaths. The direct asthma care costs incurred by the National Health Service are estimated to be more than £1 billion annually. The total annual cost to the UK economy increases to £6 billion when considering the cost of working days lost due to asthma. Severe asthmatics are responsible for about 50% of this economic burden.

1.2. Airway remodelling in asthma

There is universal agreement that the two fundamental pathological domains of asthma pathogenesis are airway inflammation, with the involvement of a wide range of inflammatory cells, and airway remodelling changes which include increased airway smooth muscle (ASM) mass, mucus gland hypertrophy, new vessel formation in the submucosa, subepithelial fibrosis due to excessive deposition of matrix proteins in the lamina reticularis, and epithelial changes including epithelial fragility and increased mucus secretion by goblet cells .(See Figure1.1). However, the relative contribution of these two domains of airway inflammation and airway remodelling to the pathogenesis of asthma or the exact nature of their relationship has not been well defined.

1.2.1. History and definition of airway remodelling in asthma

The first evidence of airway remodelling in asthma was provided by Hubert and Koessler in 1922 who described increased ASM mass in post-mortem studies of patients who

died of asthma [4]. Throughout the years, the study of airway remodelling has been hampered by researchers concentrating their efforts on studying inflammation as most believed remodelling to be just an aberrant repair process resulting from chronic inflammation. However, in recent years there has been growing renewed interest in studying airway remodelling in asthma. This is due to several factors including the continuing need for developing new asthma therapies targeting severe asthmatics where significant morbidity and mortality still exists despite novel targeted anti-inflammatory treatments. Furthermore, recent studies supporting the use of bronchial thermoplasty as an anti-remodelling therapy have helped reignite the debate on the significance and relevance of the process in asthma.

The use of the term "airway remodelling" in asthma is variable and usually context specific. While most use the term to describe the microscopic changes associated with the disease, others use it to describe the macroscopic changes seen on cross-sectional imaging or the physiological phenomenon of fixed airflow obstruction seen in some asthmatics. It is said that the disagreement in the definition of airway remodelling even includes the way the word remodelling is spelled "remodeling vs. remodelling" [8].

In most biopsy studies of asthma and in the context of this thesis, airway remodelling refers to all the non-acute histopathological changes seen on light microscopy of biopsies obtained from proximal airways. These changes include all the compartments of the airway tissue including the airway smooth muscle, the epithelium, the mucus glands and the extracellular matrix including resident cells e.g. myofibroblasts.

1.2.2. Aetiology of airway remodelling in asthma: is it all due to inflammation?

Chronic inflammation in various pathological conditions other than asthma, leads to structural changes in the tissue; as such, inflammation has long been thought as the primary cause of airway remodelling in asthma, and hence should be the target of any therapy that aims at modifying remodelling. Consistent with this view, different aspects

of remodelling have been clearly shown to be caused or worsened by inflammation. For example, subepithelial fibrosis, one of the most studied features of remodelling in asthma, has been shown to be closely associated with eosinophils-derived cytokines, namely IL-5 and transforming growth factor-*beta* (TGF-β) [9,10]. However, mounting evidence especially from studies on the paediatric asthmatic population have shown that the remodelling, at least in part, is not simply a consequence of inflammation. While airway inflammation and remodelling were shown in children aged 1-3 years with preschool wheeze, this was not seen in symptomatic infants with reversible airflow obstruction [11-13]. Supporting this, Saglani *et al.* showed, in a neonatal murine model of allergic asthma, that both epithelial and extracellular matrix (ECM) remodelling occur simultaneously [14]. This was also suggested by Kariyawasam *et al.* who, in a group of asthmatics, showed that both inflammation and remodelling occurred 24 hours after allergic challenge and only features of the latter persisted [15]. They showed on bronchial biopsies obtained pre-, and one and seven days post-challenge, sub-epithelial fibroblast number and activation, and reticular basement membrane (RBM) thickening increased and persisted over the seven days and was associated with increased airway hyper-responsiveness. In contrast, inflammation observed at day one had resolved by day seven. This suggests that remodelling occurs in response to allergen challenge; is related to airway dysfunction, might require inflammation for its initiation, but is not dependent upon persistent inflammation. More recently, a biopsy study of a group of preschool childern with severe recurrent wheeze has shown no relationship btweeen markers of inflmmation and remodelling [16].

All this supports the argument that in the natural history of asthma, airway inflammation and remodelling might occur in parallel and that aspects of remodelling can be a consequence of airway stimuli acting directly on the airway structural cells. Indeed, bronchoconstriction resulting from methacholine, a cholinergic drug normally used to measure AHR, was shown to induce remodelling in asthmatics without causing

inflammation [17]. Furthermore, viruses have also been implicated in remodelling. Viruses are the most common cause of asthma exacerbations and, therefore, are possibly involved in the induction of remodelling although the evidence is scanty. Rhinovirus *in vitro* can promote the release of matrix proteins from cultured ASM suggesting it may directly influence remodelling [18].

The existence of a close link between airway inflammation and airway remodelling in asthma is not in doubt, however the exact nature of this relationship is far from being well defined. Indeed the inflammatory cascade seen in asthma has been shown to affect and influence every airway structural cell type with an ever increasing evidence of the direct effect of various inflammatory mediators and cells, as discussed in the next section, on the all the different elements of remodelling. Inflammation creates a significant confounding factor when attempting to study any aspect of remodelling including its pathogenesis, role in natural history, and more importantly its independence from inflammation. This is further complicated by the proven immunomodulatory properties of airway structural cells [19-21].

Airway remodelling is clearly a complex and dynamic process with heterogeneous elements each one with different multifactor aetiologies and a varied role in the natural history of asthma. Inflammation is significantly linked to almost all aspects of remodelling; however, multiple aspects have been shown to occur parallel rather than consequential to inflammation. The proven limitation of current asthma anti-inflammatory treatments in preventing or reversing remodelling is further proof that the process is fundamentally significant in the pathogenesis of asthma [22]. The relation between remodelling and inflammation is complex and still poorly understood, however, in my opinion; this link between the two processes is probably heterogeneous and varies with disease phenotype, asthma severity, inflammation type and phase of the disease's natural history.

1.2.2.1. Role of inflammatory cells

Mast cells and immunoglobulin E (IgE) have a central role in the classical Th_2 allergic asthma paradigm [23]. Briefly, in allergic asthma, antigens are presented by antigen-presenting cells, which in the airway are mostly dendritic cells, to naive T cells which In turn differentiate into T helper 2 cells and start to produce IL4, IL5 and IL13. IL4 and IL13 stimulate B-lymphocytes to produce IgE which upon binding to its specific high affinity receptor (FcεRI) on mast cells, and after sensitization, leads to their activation, migration and degranulation releasing various inflammatory mediators and cytokines [24]. These include mediators of the early allergic response like histamine, leukotriene C4, prostaglandin D2 and platelet-activating factors which lead to bronchoconstriction, increased mucus production, mucosal oedema and AHR. Of note, prostaglandin D2 also contributes to the pathogenesis of allergic asthma through its action on its receptor DP_2/chemoattractant receptor homologous molecule expressed on Th_2 cells ($CRTh_2$) on Th_2 lymphocytes and eosinophils facilitating their chemotaxis and activation [25]. Mast cells also produce cytokines and chemokines like IL4, IL13, and IL5, which mediate the late allergic response mainly through their action on other inflammatory cells. Directly relevant to airway remodelling, mast cells, after activation, also synthesize both TGF-β and basic fibroblast growth factor (FGF), which are strongly pro-fibrotic, and the serine proteases tryptase, chymase, and carboxy-peptidase which act on fibroblasts leading to their proliferation and increased collagen I production. Additionally, mast cell infiltration of ASM bundles, a feature seen in asthma, has been liked with AHR, increased α-actin expression and ASM hypercontractility [26]. These infiltrating mast cells are predominantly of the chymase phenotype, which is known to strongly promote Th_2 inflammation. Although some early *in vitro* studies suggested a role for mast cells in increased ASM mass, this has not been supported by more recent clinical studies

[27,28]. Interstingly, a recent study has shown that through tryptase-dependent MMP-1 activation, mast cells are able to promote ASM prolifration and influence AHR [29].

IgE also contributes to asthma pathophysiology by acting directly on basophils and dendritic cells which also express FcεRI. Moreover, it stimulates B-lymphocytes, monocytes, macrophages, and dendritic cells though the low affinity IgE receptor (FcεRII or CD23) [30]. Although IgE may affect remodelling indirectly through the aforementioned allergic cascade, there is recent evidence to support a direct effect on structural effector cells. Roth *et al.*, using a human ASM culture, showed increased proliferation and collagen deposition upon stimulation by activated IgE [31]. Mast cells also contribute directly to epithelial remodelling. FcεRI-mediated amphiregulin production, a ligand for EGF, by mast cells is up-regulated in asthma, leads to increased mucin gene expression in epithelial cells with subsequent increased mucus production, and correlates with to goblet cells hyperplasia [32]. Moreover, *ex vivo*, mast cell derived amphiregulin has also been shown to induce the proliferation of primary human lung fibroblast and ASM cells [33,34].

Eosinophils have also been linked to airway remodelling. In animal models of asthma and in asthmatics, the association of subepithelial fibrosis and submucosal eosinophils has been well documented [35]. Cytotoxic proteins which are stored and secreted by eosinophils upon activation have been shown to directly cause epithelial damage [36]. The nature of the relationship between other inflammatory cells and airway remodelling in asthma is far less known. Th_2 lymphocytes have been linked to ASM proliferation and remodelling, however, all the evidence for this is from *in vitro* studies only [37].

1.2.3. Components, mechanisms and functional relevance of airway remodelling

1.2.3.1. Airway smooth muscle remodelling

ASM cells are normally present in most airways where they form bundles arranged geodesically around the airway lumen. As a result of this geodesic arrangement, ASM contraction leads to both narrowing and shortening of the airways. This is important in asthma where, as a consequence of remodelling, airways are stiffer and resistant to shortening hence most of the force generated from ASM contraction translates into excessive airway narrowing [38]. The physiological function of ASM is not clear. While some argue that ASM has a role in the maintenance of bronchial tone, clearance of respiratory secretions and regulation of ventilation, others consider ASM a vestigial component of the airway with no function [39,40]. Increased airway smooth muscle mass was the first remodelling feature to be observed in asthma. Huber and Koesser in 1922 reported increased ASM mass in patients who died from asthma when compared with controls that died from other conditions [4]. This has since been confirmed in patients with asthma of all severities and in small and large airways. Until the significance of inflammation had been suggested, asthma was primarily considered an ASM dysfunction.

Increased ASM mass in asthma is due to hyperplasia and possibly hypertrophy, although the evidence for the latter is incongruent [41,42]. ASM hypertrophy was shown in some studies but others failed to do so [41-43]. There is an ongoing debate regarding the origins of ASM hyperplasia in asthma. In biology, hyperplasia of any tissue is usually caused by the increased rate of division of primary cells constituting the tissue, that is, proliferation. Consequently, proliferation has initially been regarded as the main mechanism underlying ASM hyperplasia in asthmatics. However, despite extensive research, the evidence for ASM proliferation in asthmatics remains largely inconclusive. In some *ex vivo* studies [44,45], but not others [28,46], asthmatic ASM showed

increased proliferation compared to non asthmatics. More importantly, several *in vivo* studies have failed to demonstrate proliferation [41,43]. Similarly, there is a lack of evidence to suggest that *ex vivo* ASM from asthmatics has an altered rate of survival. It therefore remains unclear whether altered cell proliferation or survival contributes significantly to ASM hyperplasia in asthmatics, although this seems unlikely to be the principal underlying mechanism.

Other cells have been suggested as possible origins of ASM hyperplasia, these include tissue-resident mesenchymal stem cells (MSCs). There is some evidence to support the involvement of lung MSCs in the pathogenesis of some fibrotic lung conditions. Studies have shown that MSCs undergo transformation into a myofibroblastic phenotype in premature infants, possibly contributing to the pathogenesis of bronchopulmonary dysplasia, and in allograft lungs, possibly assisting in the development of bronchiolitis obliterans [47,48]. This suggests that lung resident MSCs can potentially transform into a smooth muscle phenotype and possibly contribute to ASM hyperplasia in asthma although the evidence is lacking. Bentley *et al*. showed increased numbers of MSCs in the lung of an ovalbumin-sensitized mouse model following aerosol challenge, although this was not linked to any of the features of remodelling [49]. Pericytes are contractile cells normally wrapped around the endothelium of most microvasculature. They have been found to fulfil both the multi-lineage and markers criteria for MSCs and many nowadays argue that pericytes could be the source of all MSCs. In kidney injury, pericytes have been found to be a source of mesenchymal contractile cells. Interestingly, pericytes might be involved in ASM hyperplasia in asthma, although the evidence is limited to only one report in the literature. In a murine asthma model, upon aeroallergen exposure, pericytes seem to detach from vasculature and migrate to the sub-epithelium of the airway, with up-regulation of α-smooth muscle actin [50]. Epithelial cells (as discussed in the section 1.2.3.4) have also been suggested to contribute to ASM hyperplasia. (See Figure 1.2).

In recent years, fibrocytes, a type of blood-derived mesenchymal progenitors, have been identified as a possible source of ASM in asthma. They were first described by Bucala *et al*. in 1994 in a mouse model of wound healing, as a distinctive group of cells with both hematopoietic and mesenchymal properties [51]. Since then fibrocytes have gained increasing prominence with emerging evidence of their involvement in the aberrant tissue repair evident in a number of fibrotic lung disorders, including pulmonary hypertension, idiopathic pulmonary fibrosis and asthma [52]. Fibrocytes normally constitute 1% of the peripheral blood leukocytes and express a wide range of hematopoietic stem cell, myeloid and mesenchymal markers, including CD34, CD11b, CD13, CD45, CXCR4, CCR7, procollagen-I, vimentin and α-smooth muscle actin [53]. After leaving the circulation and once in any tissue, fibrocytes lose the expression of their hematopoietic markers and gain mesenchymal markers accentuated by transforming growth factor-β (TGF-β) and endothelin-1, both of which are found at increased levels in asthmatic airway tissues compared to non-asthmatic tissue [54,55].

The first evidence of fibrocyte involvement in asthma was described a decade ago. Schmidt and associates showed, in a group of allergic asthmatics, an increase in the number of cells co-expressing CD34, procollagen-I mRNA, and α-smooth muscle actin in the sub-epithelium following allergen exposure [56]. The authors also showed, by labelling and tracking fibrocytes in an experimental mouse model of allergic asthma, that fibrocytes are recruited from the circulation after allergen exposure. In a study connecting fibrocytes directly to remodelling, Nihlberg *et al*. showed a clear correlation between the thickness of the basement membrane and the number of tissue fibrocytes in a small group of patients with steroid-naïve mild asthma [57]. Wang *et al*. demonstrated a relationship between peripheral blood fibrocytes numbers and airflow obstruction [58]. The increased proliferation of fibrocytes in asthmatics with chronic airflow obstruction has recently been linked to oxidative stress mediated via up-regulation of the epidermal growth factor receptor pathway [59]. The clearest evidence

of the direct contribution of fibrocytes to ASM hyperplasia *in vivo* was presented by Saunders *et al*. [60]. They showed that, compared to normal controls, severe refractory asthmatics have an increased number of fibrocytes in the peripheral circulation and also in the airway submucosa. More interestingly, they demonstrated an increased number of fibrocytes in the ASM bundle in asthmatics of all severities. It is important to note that despite the relatively large number of subjects in this study it failed to demonstrate any link between fibrocytes and lung function.

The significance of ASM contraction in asthma is obvious as it is the effector of reversible airway obstruction and AHR. Most bronchodilator therapy in asthma acts by relaxing ASM resulting in improvements in symptoms and lung function. Furthermore, there is consistent evidence that ASM in asthma is inherently hypercontractile [61]. *Ex vivo* gel-contraction assays have shown that ASM cells from asthmatics contract more than ASM from healthy donors [62,63]. Although the mechanisms driving this hypercontractility are not very clear, several have been suggested. Sutcliff *et al.* have shown increased oxidative stress burden in asthmatic ASM with excessive production of reactive oxygen species as a result of nicotinamide adenine dinucleotide phosphate oxidase type-4 (NOX4) overexpression. This was associated with airflow obstruction, AHR and ASM hypercontractility, as measured by gel-contraction assay [62]. The latter was abolished, *in vitro*, by adding an inhibitor of NOX4. Calcium signalling has also been implicated in ASM hypercontractility. Several studies have demonstrated altered calcium homeostasis in asthma with increased cytosolic calcium levels which, given the central role of calcium in ASM contraction, would potentially result in ASM hypercontractility [64]. Furthermore, as suggested by animal studies, ASM hypercontractility could also be mediated through abnormalities in the RhoA/Rho kinase signalling pathway leading to increased calcium sensitivity [65]. Finally, increased expression of the contractile protein α-actin, resulting in the transformation of ASM

cells to a hypercontractile phenotype, has been shown, *in vivo* and *in vitro* co-culture, to be closely linked to mast cell infiltration of ASM, a known feature of asthma [26].

Increased ASM mass is a structural predictor of disordered airway physiology in asthma [41,66]. Additionally, ASM is an important source of inflammatory mediators and matrix proteins. Adding asthmatic serum to ASM cells in culture was shown to result in excessive ECM protein production [67]. Several reports suggest that ASM cells in asthma are pro-inflammatory with the expression of various immunomodulatory mediators including IL-1, IL-6, IL-8 and prostaglandin E2 [68,69]. From all the above evidence it is clear that ASM is pivotal in the pathogenesis of asthma with roles going far beyond being the regulator of bronchial motor tone.

1.2.3.2. Mesenchymal fibrosis

ECM changes have been extensively investigated in asthma. The airway ECM is mainly composed of various types of macromolecules including proteoglycans, collagens, elastic fibres and other non-proteoglycan glycoproteins like fibronectin, laminin and tenascin. They are secreted mainly by mesenchymal cells and they provide structural and biological support to the airway tissue. The ECM dynamic environment is normally controlled by matrix metalloproteinases (MMPs), which are proteolytic enzymes that degrade ECM proteins; and their inhibitors termed tissue inhibitors of metalloproteinases (TIMPs).

ECM protein content in asthma is abnormal with studies showing excessive laminin, fibronectin, tenascin, proteoglycans and the collagens I, III and V; and reduced content of collagen IV and elastic fibres [70-72]. Although excessive ECM proteins deposition occurs diffusely in the airway tissue, it is most intense in the reticular lamina which is normally a thin layer in the lamina propria just below the true epithelial basement membrane. This subepithelial fibrosis is normally referred to, as in this thesis, as RBM thickness, although this is obviously a misnomer. Increased RBM thickness has been

reported in both adults and children with asthma and throughout the severity spectrum. It has been linked to airway thickness seen on CT, AHR and obstructive lung function [73-75]. Increased RBM thickness has been strongly associated with Th2 inflammatory cells and mediators. This was completely absent in murine models of allergic asthma which are depleted from IL-5 or eosinophils [76]. The action of inflammatory cells on ECM protein deposition is mediated mainly by TGF-β, which is a profibrotic cytokine that induces mesenchymal cells ECM protein production [77]. Abnormalities of both MMPs and TIMPs have been reported in asthma although their exact role in ECM remodelling in asthma is not fully understood [78].

1.2.3.3. Vascular remodelling

Bronchial vascular remodelling features in asthma include new vessel formation, increased blood flow and vascular leakage. Under a favourable pro-angiogenic environment, as occurs in asthma, the bronchial vasculature is able to proliferate by sprouting from pre-existing vessels. This submucosal neoangiogenesis is a recognized feature of remodelling in adults and children with asthma and increases with the disease severity [79]. It has been shown to be negatively associated with post-bronchodilator Forced expiratory volume in 1 second (FEV_1) and, additionally, a link with AHR has been suggested by some, but not all, studies [80,81]. Vascular endothelial growth factor (VEGF) is the most significant mediator of neoangiogenesis in asthma. VEGF is secreted by a range of cells including Th2 cells, eosinophils, macrophages and ASM cells. Increased levels of VEGF in asthma have been associated with severity and airflow limitation [81]. Anti-VEGF has been successfully used to ameliorate vascular remodelling, but only in a murine model of asthma [82].

Increased airway vascular flow in asthma is caused by both increased vessel numbers and vasodilatation induced by various inflammatory mediators. However, the functional and physiological consequences of this are not fully known. Increased permeability is

also a feature of vascular changes in asthma and is driven by various mediators including histamine, prostaglandins, leukotrines and VEGF. This leads to airway oedema and contributes to airway inflammation and obstruction [83].

1.2.3.4. The epithelium in asthma

The airway epithelium plays a crucial complex role in disease and in health as it is the only part of the airway structure which has direct contact with the external environment and through it allergens and other stimuli influence the airways. Epithelial cells have a significant modulatory influence on airway inflammation and on underlying mesenchymal changes. These influences are far beyond its function as a simple mechanical barrier [84]. Epithelial cells have a central role in linking innate and adaptive immunological responses in the normal human lung. This is mainly through activation of pattern recognition receptors (PRRs), expressed by epithelial cells, by pathogen-associated molecular patterns (PAMPs) from microorganisms or by damage-associated molecular patterns (DAMPs) usually resulting from cellular damage. Upon activation epithelial cells secrete a range a chemokines and cytokines including CCL2, CCL20, TSLP, IL-25, IL-33 and granulocyte-macrophage colony stimulating factor (GM-CSF) resulting in the attraction and activation of dendritic cells and monocytes which in turn act as antigen presenting cells to immature T-cells which then differentiate into Th2 lineage. Interestingly, early in childhood, the threshold of PRRs activation might be influenced by exposure to such stimuli as cigarette smoke or respiratory syncytial virus infection which then theoretically lead to up regulation of the epithelial response to allergen and possibly the development of allergic airway diseases, although this remains to be proven [85,86]. Airway epithelial cells can also influence lung inflammation through their apoptotic function. Juncadella *et al.*, recently, using an asthma murine model, demonstrated that epithelial cells have an apoptotic function which is dependent on the GTPase Rac1 and this has an anti-inflammatory effect medicated by IL10 [87].

In asthma the respiratory epithelium is abnormal; however, the pathological and clinical relevance of this is not yet fully resolved. Several studies have shown that the asthmatic epithelium is fragile with abnormal tight and adherence junctions. This fragility is partly inherent, as it is present in differentiated epithelial cultures which are devoid of inflammatory cells and mediators, and partly caused by elements of airway inflammation [88-90]. This defective barrier function leads to worsening of airway inflammation by enhancing the exposure of dendritic cells to allergens in the airway lumen [91]. Moreover, the asthmatic epithelium reacts abnormally to viral infections by having impaired apoptosis, a normal protective biological reaction in infected epithelial cells. This allows viruses to further replicate leading to worsening of infection [92]. Defective wound repair is also evident in the asthmatic epithelium [93]. This is associated with increased epidermal growth factor receptor (EGFR) expression which in turn is linked with excess TGF-β production which is known to exacerbate inflammation and ECM remodelling [94].

Epithelial mesenchymal transition (EMT) is another biological mechanism in asthma through which the epithelium can directly influence the cellular composition of the underlying lamina propria. EMT describes a biological process in which epithelial cells lose adhesion and polarity and migrate into the lamina propria and acquire mesenchymal cell properties like increased extracellular matrix protein production and prolonged survival. EMT is known to play a significant role in fibrotic conditions including idiopathic pulmonary fibrosis; however, in asthma the evidence for the significance of EMT is limited [95]. TGF-β, which is increased in asthma, has been shown to increase EMT in normal human epithelial cultures [96,97]. Epithelial cells from asthmatics have been shown, in culture, to undergo EMT in response to TGF-β markedly more than non-asthmatics cells [98]. Moreover, in a murine asthma model, EMT was shown to occur in response to prolonged allergen exposure [99]. Mediated by TGF-β, Eosinophils have been shown to induce EMT when co-cultured with human epithelial

cells and also when placed in the trachea of a murine model [100]. Despite all this evidence, EMT has yet to be demonstrated in asthma *in vivo*.

1.2.3.5. Mucus related changes

The respiratory airways produces mucus which is composed of heterogeneous constituents normally homogenised together in a gel-like form by the effect of containing mucins which are glycosylated proteins that play a significant role in the pathogenesis of asthma. The biochemical characteristics of mucins include a very high molecular weight and heavily glycosylation with carbohydrates forming 50-90% of their total mass. Airway mucins are generally divided into two types; membrane bound mucins which are attached to the cell membranes with a large extracellular domain; and secreted mucins which are gel-forming [101]. Secreted mucins have cysteine-rich terminal domains that, through disulfide bonds, form dimmers and then long chain polymers which give the airway mucus its viscous and elastic (gel-like) properties. In the airways, secreted gel-forming mucins are produced by luminal secretary cells, named goblet cells, and by submucosal glands which are mainly present in large cartilaginous airways. Although more than 20 mucin (*MUC*) genes have been identified in humans, only two mucins are significantly expressed in the airway mucus; MUC5AC, mainly produced by goblet cells and MUC5B which is produced by mucus glands [102].

In asthma mucus-related abnormalities include mucus hypersecreation, increased goblet cells number, enlarged mucus glands and abnormal ciliary function. From post-mortem studies mucus plugging is frequent in patients dying of status asthmaticus [103]. Increases in both mucus production and mucin genes expression, namely *MUC5AC* and *MUC5B*, are known in asthma and are associated with AHR, high airway resistance and deterioration of symptoms and lung function [104,105]. Th2 inflammation has been shown to contribute to mucus hyper-production [106,107]. Mucus hyperviscosity due to increased cells, plasma proteins, mucins and nucleic acids; together with a ciliary

dysfunction, both of which have been shown in asthma; result in a defective mucociliary clearance [108]. This has many detrimental effects in asthma including contributing to the airflow obstruction and delayed clearance of allergens and pathogens from the airway thus allowing increased epithelial exposure to those stimuli. Epithelial metaplasia resulting in goblet cells hyperplasia is known to occur in asthma and is associated with mucus hypersecreation. Goblet cells lack cilia and thus their hyperplasia reduces the epithelial area covered by cilia and consequently leads to mucus clearance delay. Mucus gland hypertrophy, although not as well studied as the other features of remodelling due to the paucity of mucus glands in endobronchial biopsies, is present in asthma and contributes to increased airway wall thickness and the airflow obstruction.

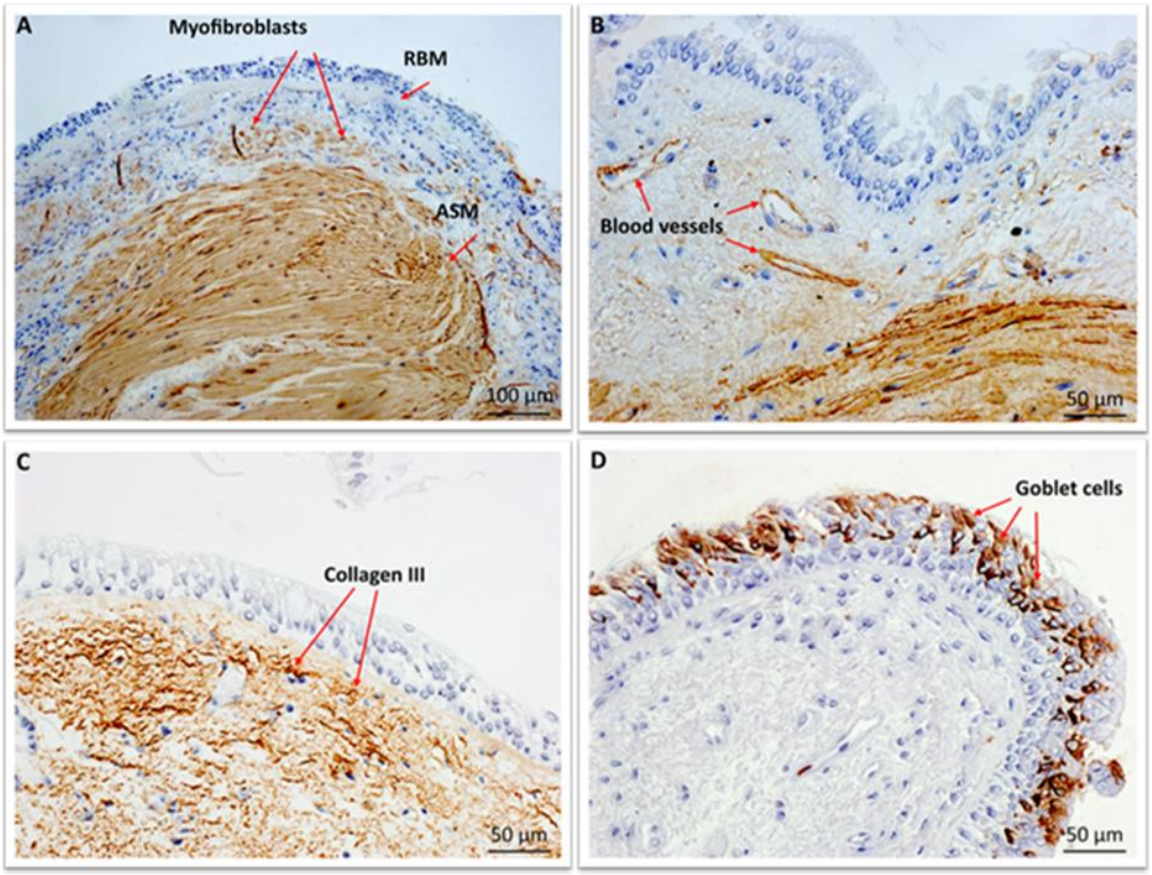

Figure 1.1: Endobronchial biopsy sections from asthmatic subjects showing features of airway remodelling

Endobronchial biopsy samples from asthmatic subjects showing features of airway remodelling. Sections **A** and **B** (stained with smooth muscle actin antibodies) show epithelial metaplasia with loss of cilia, increased ASM mass, myofibroblast hyperplasia (**A**), increased RBM thickness (**A**) and neoangiogenesis (**B**). Section **C** (stained with Collagen III antibodies) shows excessive deposition of collagen III in the lamina propria and in the reticular basement membrane. Section **D** (stained with MUC5AC antibodies) shows goblet cell hyperplasia with increased stored mucin.

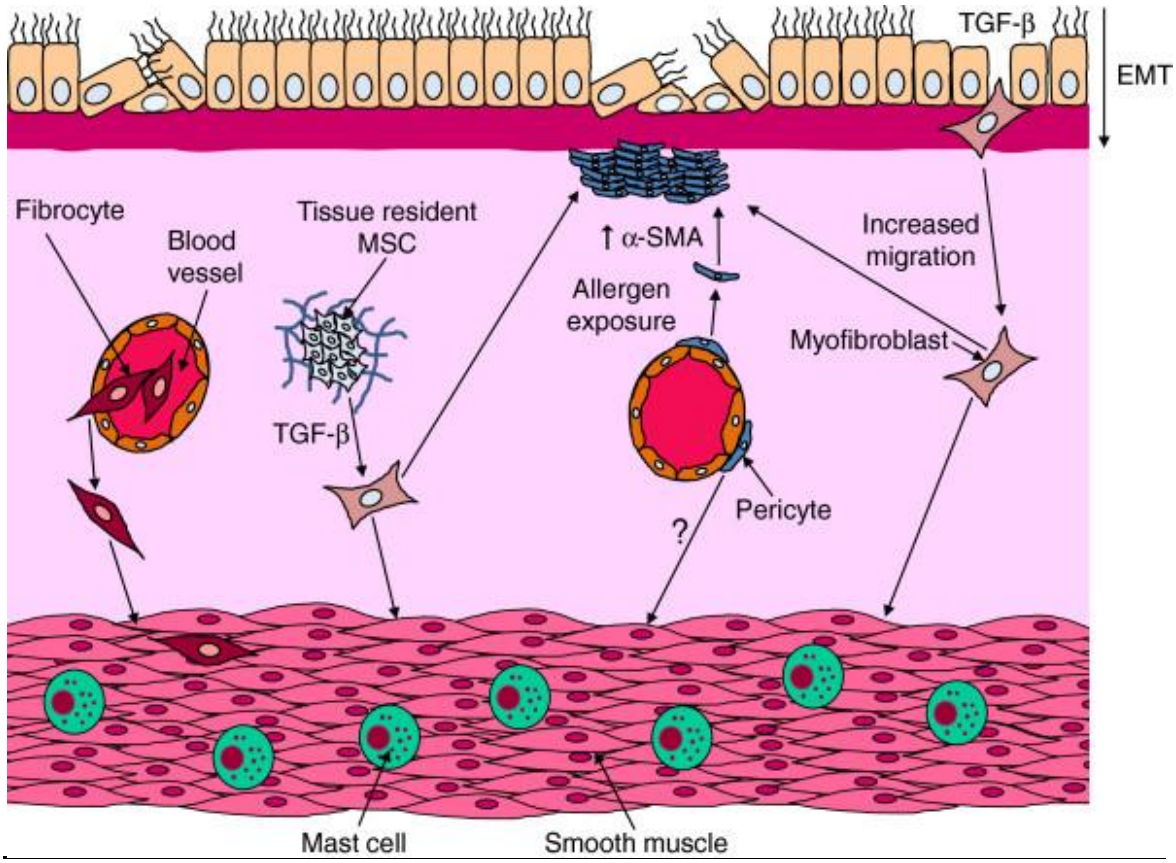

Figure 1.2: The potential origins of ASM hyperplasia in asthmatic airways.

Increased airway smooth muscle (ASM) could result from recruitment of fibrocytes from the peripheral circulation to the airway tissue; or from differentiation of tissue resident mesenchymal stem cells (MSCs); or from epithelial-mesenchymal transition (EMT) of epithelial cells. Additionally, pericytes may also contribute to this process. To transform to functioning ASM these cells lose their non-mesenchymal markers and acquire mesenchymal characteristics like increased α-SMA expression.

1.2.4. Measuring airway remodelling in Asthma

This remains one of the major difficulties in studying airway remodelling in asthma. Asthma phenotypes (a phenotype is defined as observable characteristics of a disease that are determined from the interaction between genetics and the environment) are the end result of a long chain of biological events. In this paradigm, individuals with the susceptible genotypes, under the influence of factors that affect gene expression, have molecular and cellular changes which result in histological changes, i.e. remodelling, leading to airway physiological changes and other patients' phenotypic characteristics. Consequently, in this model, remodelling is directly connected to physiology and thus one can use lung function test as a surrogate measure for remodelling, however, this connection between airway remodelling and function has been difficult to prove. Although some features of airway remodelling have been linked with AHR and airflow obstruction, direct causality remains unproven [109,110].

Biological samples used for studying remodelling, beside *ex vivo* tissue cultures and animal models of asthma, include lung tissue obtained from lung resections or post-mortems, or much smaller airway biopsies obtained through flexible bronchoscopy. Specimens obtained from post-mortems and lung resections have contributed significantly to our understanding of the disease, especially fatal asthma; however, they have obvious limitations. Surgical resections carry significant morbidity and thus, ethically, they cannot be done solely for research purposes, while post-mortem samples are scarce and of limited value. There are two types of biopsies that can be obtained through flexible bronchoscopy for the purpose of studying remodelling, transbronchial and endobronchial biopsies. Transbronchial biopsies are obtained from distal small airways and include alveolar tissue, but carry a significant risk of pneumothorax or excessive bleeding and therefore are not widely performed.

The most common histological samples obtained for studying remodelling are endobronchial biopsies (EBBs) which are also obtained using flexible bronchoscopy but from large proximal airways under direct bronchoscopic vision [111]. They are comparatively safe even in severe asthmatics and can be used to assess the dynamics of remodelling and inflammation in the context of asthma drug trials. Nonetheless, EBBs also have significant limitations to be considered when used in measuring airway remodelling. These include the inability of EBBs to represent changes in the whole airway, especially small airways which have a crucial role in asthma pathogenesis, as EBBs are only obtained from the surface of carina of large airways; large intra-subject variability which is also affected by the sampling process and finally the possibility of reference space bias [112]. The lack of standardized guidelines for the methodology of assessing airway remodelling in biopsies creates another source of bias. Some measures of remodelling like reticular basement membrane (RBM) thickness have been well established, as most studies use the method described and validated by Sullivan *et al*, while other measures have been less well defines e.g. RBM-ASM distance [41,113]. Stereological methods have been suggested for cell counts, however they are complicated, time consuming and their superiority is less proven when using EBBs. Bronchoalveolar lavage (BAL) is very useful in assess inflammatory changes in asthma; and although remodelling mediators in asthma have been studied in BAL, its value in measuring remodelling is limited [114,115]. The same principle also limits the value of using induced sputum and exhaled breath condensate, beyond measuring mediators, to assess remodelling [116,117].

Non-invasive imaging techniques for measuring airway remodelling in obstructive lung diseases have recently gained significant interest. The modalities of imaging involved include computed tomography (CT), discussed in the next section, and Optical Coherence Tomography (OCT) [118]. In OCT, near-infrared light is used to generate cross-sectional images. Compared to ultrasound, it uses shorter wavelength and greater

frequency thus giving sections with much higher resolution (10-20 μm) although with lower tissue penetration (2-3 mm) [119]. This theoretically would allow OCT to detect microscopic tissue changes e.g. in ASM or RBM. OCT has been well established in cardiovascular research; however, its role in airway diseases is still emerging. Caxon *et al.,* in a group of smokers (n=44), demonstrated that OCT was superior to CT in detecting changes in the airway wall that better correlated with changes in spirometry [120]. In asthma, a recent report showed that OCT performed in patients undergoing bronchial thermoplasty was able to detect ultrastructural changes in airway remodelling including reduction in ASM mass [121]. Although these results are very exciting, they are only preliminary and more studies on the use OCT in airway diseases are needed.

1.3. Using computed tomography to measure airway remodelling

1.3.1. History, physics and applications of Computed Tomography

Computed tomography (CT), also known as computed axial tomography (CAT), scanning is an X-ray based investigational technique widely used in almost all medical specialities. X-radiation, a form of electromagnetic radiation, was first discovered by the Dutch physicist and engineer Wilhelm Röntgen in 1895 when by chance he stumbled on a new unknown radiation, hence the name *X-rays*, that was being emitted when he was passing an electrical discharge though a vacuum glass tube. He rapidly realised that this new radiation could pass though different structures including the human body and was able to cast shadows of solid materials including bones [122]. His discovery received wide international publicity and only a few months after his initial findings x-rays were used to diagnose fractures and locate bullets and shrapnel in wounded soldiers.

The advancement of our understanding of the properties of x-rays, together with new developments in the field of computation technology, have led to the invention of CT by British scientist Godfrey Hounsfield in 1972. CT scanning is based on the ability to assess

various tissue densities by calculating their attenuation coefficients, a measure of penetrability of tissues by radiation. In simple terms, the x-ray photon beams are produced by a number of emitters on the tomography which then pass through the scanned part and are received on the other side by detectors which, dissimilar to projection radiographs, do not produce an image but rather measure the attenuation of the x-ray beam. Complex mathematical computational equations are then applied to this raw data to reconstruct cross sections (tomograms) [123].

Chest CT is the principle ultimate cross-sectional imaging technique used to investigate lung diseases. It is used to diagnose almost all structural lung disease including emphysema, interstitial lung diseases, bronchiectasis, lung malignancy and pulmonary vascular diseases. Magnetic resonance imaging (MRI), the other cross-sectional imaging modality widely used in the clinical setting and often compared to CT, has major limitation when imaging the lungs due to its low-proton-density and also due to the high susceptibility of MRI to artefacts when imaging tissues with air-tissue interfaces like the lungs.

1.3.2. Clinical use of computed tomography in asthma

CT scans are commonly performed in patients with severe asthma as part of their clinical and imaging assessment. It is usually done either to exclude chronic lung condition that might present with asthma-like symptoms, like proximal airway obstruction; or to diagnose asthma-associated lung conditions such as allergic bronchopulmonary aspergillosis and bronchiectasis [124]. These scan are usually assessed by trained radiologist and expressed qualitatively. Bronchiectasis remains the most significant reported abnormality in severe asthmatics (31-40%) and is associated with the degree of airflow obstruction and the duration of the asthma [125,126]. Bronchial wall thickening, arguably an earlier stage in the natural history of

bronchiectasis, is the most common CT abnormality reported in severe asthmatics and is not only associated with disease duration but also with severity [126].

1.3.3. Association of quantitative CT (QCT) and biomarkers of asthma

The development of multi-detector CT scanners which are able to acquire detailed images of the lung in a single breath, coupled with the rapid advancements in post-acquisition processing software technology have led to the ability to produce highly representative three-dimensional reconstructions of the airway and the lung parenchyma. This has allowed direct detailed quantitative analysis of the large airways, with measurements of airway thickness and luminal area, and indirect analysis of the small airways through the comparison of lung volume and densitometry in inspiration and expiration [124].

Although several studies have shown significant correlation of QCT metrics and the various clinical and physiological markers of asthma, the role of QCT in asthma is remains largely limited to research with little use in the clinical assessment and management of patients. Increased proximal airway thickness and reduced luminal area, both representing large airways changes seen in asthma; and increased air-trapping, a measure of small airway disease in asthma, all have been found to correlate with various asthma functional markers including air flow obstruction and asthma severity. Aysola *et al* showed that proximal airway thickness was significantly higher in severe asthmatics compared to non-severe asthmatics and healthy controls and that this inversely correlated to the degree of airflow obstruction [127]. This was demonstrated by other QCT asthma studies albeit not consistently [74,128,129].

Unlike proximal airways, small airways, which play an important role in the pathophysiology of asthma, cannot be measured directly by QCT and their assessment is replaced by the measurement of air trapping indirectly by assessing lung density and volume. Several small airways markers have been developed including measuring the

mean lung density or volume during expiration and inspiration and expressing this as a ratio with higher values indicating more air trapping. Another method is measuring the proportion of lung density below a certain predefined density threshold [130,131]. Air trapping markers have been well validated and were found to correlate with asthma duration, severity, lung function and risk of hospitalisation [124,130-132].

1.3.4. QCT and airway remodelling in asthma

The current evidence evaluating the link between microscopic airway remodelling and QCT metrics in asthma is very limited, scarce and difficult to compare due to the lack of standardisation of the ever-evolving QCT techniques. Most studies are single-centred and almost all use different QCT acquisition protocols and analysis software. Variation in biopsy collection and analysis methods complicates the issue of standardisation even further.

A few clinical studies have attempted to compare QCT with bronchial biopsies. All of these studies looked at proximal airway changes and none included small airway markers. Aysola *et al* all found a strong correlation of airway thickness and epithelial thickness. Some, but not all, studies found a relation between proximal morphometry changes and RBM thickness [74,129,133]. Only one study found a link between proximal airway changes and ASM remodelling, arguably the most important remodelling feature in asthma. However, this was a small study with n=11 and included none asthmatics [134]. See Table 1.1.

Table 1.1: Clinical studies comparing quantitative CT changes with airway remodelling measured on biopsies in asthma.

Study	N	Results	Limitations
Kasahara *et al*. [135]	n=22 adult asthmatics	WA% and WT% were strongly correlated with RBM thickness	Semi-quantitative morphometry. No densitometry.
Saglani *et al*. [129]	n=27 Difficult asthmatics Median age = 10.5	No correlation between WT% and RBM thickness	Semi-quantitative morphometry. No densitometry.
de Blic *et al*. [133]	n=37 severe asthmatics Median age = 10.5	WT correlated RBM thickening (r = 0.34; P = .04), FeNO (r = 0.45; P = .02) and ECP (r = 0.4; P = .05)	Semi-quantitative morphometry. No densitometry.
Montaudon *et al*. [134]	n=11 9 asthmatics 2 healthy (retrospective)	Maximal local slope WA/LA and/or WA/TA ratios and ASM area	Small number, CT-derived variables not clinically or physiologically validated. No densitometry.
Aysola *et al*. [127]	n=32 24 asthmatics (15 severe and 9 mild) and 8 healthy	WA% and WT% correlated with epithelial thickness	No densitometry. Epithelial thickness was the only remodelling feature examined

Abbreviations: ECP, eosinophilic cationic protein; LA, lumen area; FeNO, exhaled nitric oxide, RBM, reticular basement membrane; TA, total area; WA, wall area; WA%, wall area percentage; WT, wall thickness; WT%, wall thickness percentage.

1.4. Effects of asthma therapy on airway remodelling

Understanding the effect of therapy on various pathophysiological aspects of a disease occasionally helps to define their role and relevance. A good example in asthma is the advancement in our understanding of the role of eosinophils in causing asthma exacerbations though studies involving targeted anti-eosinophilic therapies namely clinical studies of the anti-IL5 mepolizumab [136]. Using the same approach studying the effect of proven and experimental asthma therapies on airway remodelling might help in understanding the significance of process.

1.4.1. Corticosteroids

With the exception of patients with mild disease, where only as-needed inhaled short-acting β2-agonists suffice to control symptoms, glucocorticoid (GCs) therapy remains the cornerstone of asthma management both in stable disease state and in exacerbations. The first controlled trial showing the effectiveness of GCs in improving asthma symptoms dates back to the 1950s. Inhaled corticosteroids (ICS) were first introduced in the early 1970s and since then they remain the most significant preventative therapy for asthma as recognised by all current guidelines [137]. Arguably, since the introduction of ICS, no new asthma therapy has had a similar transfiguring effect on chronic asthma, and hence ICS are the benchmark against which all new therapies are compared in both efficacy and adverse events profiles.

GCs have been clearly shown to ameliorates asthma symptoms, improve lung function and reduce exacerbation. GCs main mode of action in asthma is through their anti-inflammatory effect. This is mainly through their direct effect in different white blood cells including T-cells, mast cells and eosinophils. GCs suppress these leukocytes by inhibiting chemotaxis and adhesion thus reducing recruitment, reducing production of inflammatory cytokines, inhibiting phagocytosis and inducing apoptosis. Airway

structural cells, as will be discussed, are also influenced by GCs and that might also be contributing to the therapeutic effect. In general GCs exert their action through influencing transcription factors which are proteins that bind to specific promoters regions of the DNA called glucocorticoid response elements (GREs), and thus control gene expression [138]. Initially GCs cross the cell membrane and bind with a specific intracellular glucocorticoid receptor. This leads to translocation of the receptor to the cell nucleus where it undergoes dimerization and then binds to corticosteroid responsive genes altering their transcription and subsequently influencing protein synthesis. This process is called transactivation. Transactivation has a major role in metabolic homeostasis like the regulation of glucose and lipid metabolism [138]. Furthermore, activated glucocorticoid receptor can also alter gene expression indirectly, in an inhibitory process termed transrepression, by combining, as a monomer, with other transcription factors such as AP-1, NF-k-B and *smad3* and thus preventing them from binding with their corresponding genes. Examples of genes encoding for pro-inflammatory proteins and are inhibited through transrepression include tumour necrosis factor-*alpha* (TNF-α), IL-1β, GM-CSF and nitric oxide oxidase. Relevant to airway remodelling, GCs, through the action on smad3, inhibit the TGF-β-mediated deposition of matrix proteins such as collagen, fibronectin, elastin and proteoglycans and inhibition of various metalloproteinases thus reducing TGF-β pro-fibrotic effect.

In recent years it has become more apparent that GCs can also exert effect through non-transcriptional non-genomic mechanisms, although the significance of this in asthma therapy remains to be explored [139]. Non-genomic effects are rapid in onset, taking only seconds to start, and last only for a short period, thus their role might be more beneficial in asthma exacerbations. One important example is the inhibitory action on phospholipase A2 resulting in the reduction of arachidonic acid, the precursor for prostaglandin D2 and cysteinyl leukotrienes which are important inflammatory and bronchoconstriction mediators in asthma. Another non-genomic action is through

combining with membrane bound glucocorticoid receptors leading to inhibition of T cell activation [140]. GCs can also alter ions movement through the cell membrane which might inhibit mast cell degranulation as shown on an animal model [141]. There is currently a wide consensus that the beneficiary anti-inflammatory effect of GCs results mainly from transrepression and non-transcriptional mechanisms while side effect are mainly mediated by transactivation [138,142-145].

The effect of GCs on airway remodelling is complex and still not well understood. This is despite the fact that most of the studies investigating the effect of asthma therapy on remodelling involved GCs. (See Table 1.2 and Table 1.3). As the main anti-inflammatory drugs used in asthma, and believing the simplistic paradigm of "remodelling is the result of an aberrant response to inflammation", GCs were clearly the ideal candidate to study remodelling in asthma and explore its relation to inflammation. The influence of GCs on airway remodelling is not only attributed to the effect on inflammatory cells, but, as evidence supports, is also due to mechanisms involving direct action of GCs on non-inflammatory effector structural cells and various cytokines.

1.4.1.1. GCs effect on extracellular matrix remodelling

Due to the ease of assessing ECM remodelling by measuring RBM thickness in EBBs, this has been extensively studied in the context of GCs therapy in asthma. GCs can potentially affect ECM protein deposition indirectly by their action on inflammatory cell, such as eosinophils, and inflammatory mediators which are known to play an effector role in the process. They can also affect ECM remodelling directly by acting on the regulators of ECM protein degradation MMPs and TIMPs, although the evidence for this is scarce [146,147]. However, studies have produced conflicting results with some showing reduction of RBM thickness with GCs therapy whilst others have not. Ward et al., in double-blind randomized controlled trial (RCT), demonstrated mean reduction in RBM thickness of 1.9 ABBPMcEr µm following 12 months treatment with high dose

inhaled fluticasone propionate [148]. This improvement was not seen with three months of treatment. Using a multiple regression model, the authors concluded that this reduction in RBM thickness at 12 months contributed significantly to the improvement of AHR. Sont et al. compared treatment targeted at reducing AHR to guidelines-based treatment alone in 75 mild-moderate asthmatics. The AHR-guided treatment group received a significantly higher ICS dose and this was associated with significant improvements in exacerbations, lung function and RBM thickness [149]. Other studies showed RBM thickness reduction with high dose ICS after a shorter duration of treatment [150-153]. Nonetheless, multiple studies failed to show any significant RBM thickness effect from GCs treatment (See Table 1.3) [154-157].

1.4.1.2. GCs effect on the asthmatic epithelium

Whether GCs improve or even worsen epithelial damage and other epithelial abnormalities in asthma remains a matter of disagreement with contradicting evidence. Although some studies have shown amelioration of some of the epithelial changes, not a single study has shown complete restoration of the epithelium to its normal state in response to any asthma therapy. Dorscheid *et al*. used a healthy murine model to show that treatment with dexamethasone results in increased epithelial apoptosis and shedding [158]. Furthermore, the authors also demonstrated that epithelial damage caused by allergen exposure in sensitized mice did not improve with dexamethasone treatment. This epithelial apoptotic effect of GCs was also shown in several studies using epithelial cultures; however, this is yet to be shown to have a significant effect in vivo in asthma [159,160]. The effect of GCs on epithelial repair has also been studied on animal models and using epithelial cultures. Using guinea pig tracheal epithelium, budesonide was shown not to affect epithelial repair [161]. Moreover, in a wound repair cultured airway epithelial model, dexamethasone was shown to improve the repair potential significantly compared to salbutamol-treated or control cultures [162]. On the other

hand, in another in vitro study, Dorscheid *et al*. showed that GCs impaired epithelial cell migration and wound repair after mechanical injury. Recently, this was further supported by a study showing that corticosteroid impairment of epithelial repair is medicated through glucocorticoid-induced leucine zipper (GILZ). GILZ, which was shown to be induced by dexamethasone, is known to have an anti-inflammatory effect, however, in this study, it was shown to impair epithelial repair through the inhibition of the mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK-ERK) signalling pathway [154,163]. Amphiregulin expression by mast cells, which results in goblet cells hyperplasia, is not affected by GCs therapy [33].

Although *in vivo* studies of effects of asthma therapies on the airway epithelium are rare, most studies assessing GCs have shown a beneficial effect. In a group of 6 severe asthmatics 10 years of treatment with ICS resulted in improvement in epithelial damage [154]. Similar results were seen with 3 months treatment with inhaled budesonide [164].

1.4.1.3. GCs effect on ASM

GCs have been shown to reduce human ASM proliferation directly *in vitro* [165]. Several cellular mechanisms have been suggested to drive this effect of GCs including the stimulation of p21 gene expression, an important regulator of cell cycle progression, and the inhibitory action on both cyclin D1 expression and retinoblastoma protein phosphorylation [166,167]. The inhibitory effect of GCs on ASM proliferation was shown to be defective in asthma due to an abnormal interaction between C/EBPα and the glucocorticoid receptor [168]. In culture, fluticasone propionate, through its effect on NF-κB signaling, has been found to prevent myofibroblast differentiation, a possible precursor of ASM cells [169]. In a murine asthma model, Leung et al. showed that intra-tracheal administration of ciclesonide or fluticasone, both powerful GCs, resulted in the

inhibition of features of inflammation and remodelling including ASM hyperplasia [170]. However, GCs altering ASM remodelling in asthma *in vivo* is yet to be shown.

1.4.1.4. GCs effect on Vascular remodelling

Unsurprisingly, GCs have been shown to improve all aspects of vascular remodelling in asthma including increased blood flow, neoangiogenesis and vascular leakage [171]. Orsida *et al*. showed that asthmatics on high dose ICS had reduced numbers of vessels/mm^2 of lamina propria [172]. Hoshino *et al*. further supported this in a placebo-controlled study of the effect of 6 months treatment with inhaled beclomethasone dipropionate (BDP) 800µg daily, or placebo in a group of 28 asthmatics. Beside improvements in FEV_1 and AHR, BDP treatment also resulted in a significant reduction in vessel number and percent vascularity in the lamina propria compared to placebo [173]. These vascular improvements with ICS treatment are dose dependant and have been shown in other studies using high doses of ICS (See Table 1.3) [150,174,175]. This reduction in vascularity is thought to be medicated by the effect of GCs on vascular endothelial growth factor (VEGF) expression in the airway tissue [82].

1.4.2. Other conventional and experimental asthma therapy and airway remodelling

1.4.2.1. β2-adrenergic receptor agonists

Studies examining the effect of β2-adrenergic receptor agonists on airway remodelling are lacking. Long acting β2-adrenergic receptor agonists (LABAs) persist in the airway tissue for long periods (about 12 hours), due to their lipophilic nature, and they induce sustained bronchodilatation. LABAs are always used in combination with ICS in asthma and are never used as monotherapy due to their proven association with severe exacerbations and asthma death [176]. Repeated bronchoconstriction *per se* has been proven to result in epithelial and ECM remodelling, thus LABAs, by acting as bronchodilators, could potentially prevent that [17]. Todorova *et al*. showed *in vitro* that

the combination of formoterol, a LABA, and budesonide resulted in the total inhibition of serum-induced proteoglycan production by human lung fibroblasts. Formoterol on its own had no effect at all on the process while budesonide alone inhibited proteoglycan production by 44% only [177]. This synergetic effect of LABAs and GCs on ECM remodelling was shown to be mediated by their combined effect on metalloproteolytic balance [178].

1.4.2.2. Leukotriene modifiers

Leukotrienes are inflammatory mediators that play an important role in the pathogenesis of asthma. They are lipid eicosanoids derived from arachidonic acid though the enzymatic action of 5-lipooxygenase. Although leukotrienes are mostly produced by myeloid cells, other cells are capable of up-taking the intermediate leukotriene LTA_4 and transforming it into other biologically active leukotriene e.g. LTC_4 in a process called transcellular biosynthesis [179]. The effects of the cysteinyl leukotrines (CysLT) LTC_4, LTD_4, and LTE_4 in asthma are mediated mainly through the G-protein coupled receptors $CysLT_1$, which when activated lead to eosinophilic inflammation, bronchoconstriction, mucus production and airway oedema [180]. Furthermore, the non-CysLT LTB_4, through different receptors, has also implicated in asthma with roles in leukocyte chemotaxis and AHR [181,182].

Although leukotrienes are important effectors in the inflammatory component of asthma, their role in airway remodelling is less clear. *In vitro* LTC_4 has been shown to increase rat lung fibroblast collagen synthesis [183]. This profibrotic effect has also been suggested by the finding of increased CysLT levels in idiopathic pulmonary fibrosis patients [184]. Moreover, from both animal and *ex vivo* studies, there is evidence of CysLT promoting ASM proliferation through augmenting the mitogenic effect of EGF and insulin-like growth factor [185,186].

Leukotriene modifiers are now well established in the management of asthma. Three such drugs are currently approved by the Food and Drug Administration (FDA), montelukast and zafirlukast, both $CysLT_1$ antagonists, and zileuton, a 5-lipooxygenase inhibitor. In asthma they have been shown to improve eosinophilic inflammation, lung function, AHR, asthma control and exacerbations. However, as recognised by most current guidelines, ICS are clearly superior in efficacy; hence leukotriene modifiers current role is mainly as an add-on therapy to ICS [187-189]. Studies assessing the effect of leukotriene modifiers on airway remodelling are rare (Table 1.3). Montelukast improved epithelial desquamation in animal models [190]. Henderson *et al.*, using a murine asthma model, showed that montelukast, in addition to its anti-eosinophilic effect, reduced features of remodelling like mucus plugging, ASM hyperplasia and subepithelial fibrosis [191]. Montelukast was even shown reverse allergen-induced airway remodelling in another murine model [192]. More recently montelukast has been shown to reduce ECM protein deposition in the small distal airways in sensitized guinea pigs [193]. However, the direct effect of leukotriene antagonists on airway remodelling in asthma has yet to be studied in asthmatics.

1.4.2.3. Methylxanthines

Methylxanthines exert both a bronchodilator and anti-inflammatory effects on the airways and are used in asthma mainly as an add-on maintenance therapy option and occasionally in the acute treatment of severe asthma exacerbations where they are given intravenously under close monitoring usually in the intensive care unit. The exact mechanism of action of methylxanthines is not fully understood, although the non-specific inhibition of phosphodiesterase enzyme is strongly suspected of driving most of the clinical therapeutic effects. Inhibiting phosphodiesterase type VI isoenzyme has been shown to relax human ASM and also to have a direct anti-inflammatory effect [194,195]. In asthma, methylxanthines were shown to reduce inflammatory cells and

improve both lung function and AHR [196-199]. Moreover, methylxanthines also increase corticosteroid responsiveness through their stimulatory action on histone deacetylase-2 [200]. However, the use of methylxanthines in asthma has always been limited by their significant adverse event profile and narrow therapeutic index.

The effect of methylxanthines on airway remodelling is unknown. Phosphodiesterase antagonism is unlikely to have any direct effect on airway remodelling. Although methylxanthines have been shown to reduce eosinophils, which are important effector cells in airway remodelling, no study, in our knowledge, has studied the effect of methylxanthines on airway remodelling in asthma [196].

1.4.2.4. Cholinergic antagonists

Short acting anticholinergics have an established role in the treatment of asthma. Acetylcholine is the principle autonomic parasympathetic neurotransmitter released by postganglionic neurones in the airways. Through its action on the muscarinic acetylcholine (M3) receptors located on ASM cells, mucus glands and vascular endothelium; acetylcholine leads to ASM contraction, increased mucus secretion and airway oedema, all of which directly contribute to airflow obstruction [201]. Excess acetylcholine results not only from an increased parasympathetic tone, a known feature of chronic asthma, but is also secreted by inflammatory and epithelial cells. Beyond the acute effects of acetylcholine described above, muscarinic stimulation also plays a role in airway remodelling in asthma. *In vitro* prolonged activation of muscarinic receptors has been shown to increase contractile protein expression and proliferation of primary cultured fibroblasts [202-204]. M3 receptor activation has also been shown to potentiate the proliferative effect of pro-mitogenic factors on cultured ASM [203].

Most of the studies on the effects of anticholinergics on airway remodelling in asthma have used tiotropium, a long acting Antimuscarinic with an established role in chronic obstructive pulmonary disease (COPD) and which has recently been shown to be

beneficial in asthma. In two large placebo-controlled RCTs involving severe asthmatics, tiotropium given in addition to high dose ICS and a LABA, resulted in significant improvement in FEV_1 and prolongation of time to severe asthma exacerbation [205,206]. Furthermore, in patients with milder asthma, tiotropium was also shown to improve lung function and symptoms and was comparable to LABAs [207].

The effect of tiotropium on airway remodelling has been mainly studied in animal models of asthma. Gosens *et al.*, using a guinea pig model of allergic asthma, demonstrated that administration of inhaled tiotropium during ovalbumin sensitization significantly prevented the development of features of ASM remodelling seen in the non-treated animals including increased ASM mass, increased contractile protein expression and hypercontractility [208]. Using the same guinea pig asthma model, Bos *et al.*, in additions to showing similar effects on ASM remodelling, showed that tiotropium also prevented mucus gland hypertrophy and reduced the numbers of both goblet cell, as measured by (MUC5AC)-positive cells, and eosinophils [209]. Ohta *et al.* showed reduction in all the following: airway inflammation, Th2 cytokines and TGF-β levels, ASM mass, airway fibrosis, goblet cell metaplasia and AHR; in murine chronic asthma model following treatment with tiotropium [210]. Though only *in vitro*, tiotropium has resulted in reduction of collagen synthesis by primary human lung fibroblasts [211]. It has also been suggested that it reduces respiratory syncytial virus replication in epithelial cells with possible attenuation of the inflammation and remodelling that results from the viral infection [212].

Tiotropium has been shown to reduce lung function decline in COPD, a disease where structural changes in the airways play a significant role in the pathogenesis. This clearly shows a potential role for anticholinergics beyond simple bronchodilatation. However, in asthma, the effect of anticholinergics on airway remodelling, whether directly or

indirectly through their anti-inflammatory action, is not fully explored and clearly needs further studies.

1.4.2.5. Anti-IgE therapy

As discussed earlier IgE has a central role in the pathogenesis of allergic asthma. Corticosteroids, despite having a broad anti-inflammatory action, do not reduce IgE production by B-lymphocytes and can even increase circulatory IgE levels [213]. Omalizumab, the only licensed biological treatment for asthma, has been shown in several RCTs to reduce asthma exacerbations, improve asthma related quality of life and decrease corticosteroid requirements [214,215]. Omalizumab is a recombinant monoclonal IgG1 anti-IgE antibody that works by binding to the Fc region on the IgE which normally binds to the IgE receptor FcεRI on mast cells. This means that it only binds and deactivates circulating IgE and does not affect mast cell-bound IgE. In several studies omalizumab treatment led to significant improvement of airway inflammation with reduction of a range on inflammatory cells in the submucosa including eosinophils, basophils, T-lymphocytes and B-lymphocytes. Furthermore, it was also shown to improve both the early and late allergic responses [216,217]. Some of the anti-inflammatory effect of omalizumab is independent of mast cells as it down-regulates FcεRI expression not only on mast cells, but also on basophils and dendritic cells [218,219]. Although the effect of omalizumab on inflammation is significant, the evidence for its influence on the other aspects of the asthma including remodelling has been either disappointing or not well defined. Studies examining the effect of omalizumab treatment on AHR have shown mixed results, while the improvements in lung function have been disappointing [216,220]. Omalizumab leads to the reduction of a number of remodelling mediators like endothelin-1, TGF-β and TNF-α, however, its exact effect on remodelling is not fully known. Roth *et al.* showed that IgE-induced ASM proliferation and collagen production, in ASM culture, were completely inhibited by the

drug [31]. This provided the possible biological mechanisms explaining the results of an earlier study looking at the effect of 16 weeks omalizumab treatment on right apical segmental bronchus thickness as measured by CT. In addition to improvement in sputum eosinophils, omalizumab treatment (n=16) significantly decreased percent wall area and wall thickness and increased lumen area compared to placebo (n=14) [221]. An effect of omalizumab on mesenchymal changes was also demonstrated by Riccio *et al*. who reported that, in a small group of patients with severe persistent allergic asthma, 12 months of omalizumab treatment led to a significant mean reduction of RBM thickness [222].

Treatments directed against IgE low affinity CD23 receptor have not gone beyond early phase studies; hence, their clinical efficacy or effect on airway remodelling remains to be investigated [223].

1.4.2.6. Interleukin-5 antagonists

Given the central role of IL-5 in eosinophil biology, targeting this cytokine has been regarded as logical option in various eosinophilic diseases including asthma. IL-5 is the most significant cytokine in the modulation of eosinophils; hence it is also known as eosinophil colony-stimulating factor, as it is important for all aspects of eosinophil survival and function. IL-5 is produced by Th2-lymphocytes, basophils, eosinophils and natural killer cells. It is essential for eosinophil proliferation, differentiation, maturation, chemotaxis, migration and survival in the tissues. Early clinical trials with the anti-IL-5 monoclonal antibody mepolizumab have been disappointing largely due to failure to select patients with the appropriate asthma phenotype i.e. eosinophilic asthmatics [224-226]. Later studies examining the effect of mepolizumab in patients with severe eosinophilic asthma have shown significant clinical benefits [227,228]. The largest study, the multicenter DREAM trial, has shed more light on the role of eosinophils in asthma. One year of treatment with mepolizumab reduced severe asthma exacerbations by

more than 50% [229]. A more recent meta-analysis confirmed that mepolizumab, in severe eosinophilic asthma, significantly ameliorates asthma-related quality of life and reduces exacerbations [230]. Mepolizumab has profound effect on blood, submucosal and sputum eosinophil counts [231].

Anti-IL-5 affects remodelling in asthma through its anti-eosinophilic action [232]. Using a murine asthma model, Tanaka *et al.*, showed that administration of anti-IL-5 prior to allergen exposure led to the prevention of TGF-mediated peribronchial and subepithelial fibrosis [233]. There are only a few clinical studies which examined the effect of anti-IL-5 on remodelling in asthma. In a randomised controlled trial (RCT) involving atopic asthmatics, Flood-Page *et al.* showed that, compared to placebo, 3 infusions of mepolizumab resulted in the reduction of tenascin, lumican, and procollagen III deposited in the subepithelial area with improvement of RBM thickness. There was also reduction in the profibrotic mediator TGF-β in the bronchoalveolar lavage and reduction of its corresponding mRNA from eosinophils [9]. Haldar *et al.*, after 12 months of treatment with mepolizumab, showed, beside improvements in exacerbations and eosinophil counts in blood and sputum, reduction in total airway area and airway wall area on CT compared to placebo which, interestingly, returns to the pre-treatment baseline 1 year post mepolizumab cessation [227,234]. Reslizumab is another anti-IL-5 monoclonal IgG antibody currently evaluated in phase III asthma trials. Other methods of antagonising IL-5 include anti-IL-5 receptor antibodies (anti-IL-5Rα and IL5-βc) and small interfering RNA techniques; however the effect of these treatments on airway remodelling is still unknown.

1.4.2.7. Anti-IL-13, anti-IL-17, anti-TNF-alpha and tyrosine kinase inhibitors

Interleukin-13 is a pleiotropic Th_2 cytokine that plays a significant role in the development of airway inflammation and remodelling in asthma. It is produced by activated Th2 cells, dendritic cells and mast cells, and induces goblet cell hyperplasia, IgE

production by B-lymphocytes and ECM protein deposition through fibroblast activation [235]. In several animal models anti-IL-13 has clearly been shown to improve Th_2 inflammation, airway remodelling and AHR [236-238]. In a large doubled-bling RCT (n=219) in patients with poorly controlled asthma, using lebrikizumab, a humanized antibodies that directly deactivates IL-13, has been shown to improve lung function [239]. This effect was more pronounced in patients who had high serum levels of periostin, an ECM protein produced by fibroblasts and epithelial cells under the influence of IL-13. Periostin affects epithelial cell adhesion, thus contributing to epithelial fragility; and also leads to increased activation of lung fibroblasts leading to mesenchymal fibrosis. Tralokinumab, another anti-IL-13 antibody, has been shown in a phase II RCT to improve FEV_1 when given to moderate-to-severe uncontrolled asthmatics [240]. The signaling of both IL-4, another Th2 cytokine with important remodelling and inflammatory effects in asthma, and IL-13 could be inhibited downstream by targeting the alpha subunit of the interleukin-4 receptor (IL-4α) which is a common overlapping part of both IL-4 and IL-13 receptors [241,242]. Recently, Wenzel *et al.* showed that treating patients with persistent moderate-to-severe eosinophilic asthmatics with dupilumab, a human monoclonal antibody to IL-4α, in an ICS and LABA withdrawal study, resulted in improved lung function and the reduction of both Th2-associated markers and asthma exacerbations [243].

Interleukin-17 is a cytokine mainly produced by Th17 cells, specialised lymphocytes which are distinct from Th1 and Th2 cells; and contributes to adaptive immunity especially against extracellular bacteria. Furthermore, IL-17 is also a pro-inflammatory cytokine involved in the pathogenesis of different autoimmune and chronic inflammatory conditions including asthma. IL-17 is involved in the recruitment and activation of both neutrophils and eosinophils. In asthma, IL-17 expression is increased and is associated with disease severity and AHR. Moreover, IL-17 might also contribute to remodelling in asthma through its direct action on structural cells as suggested by

some *in vitro* studies showing an effect on epithelial proliferation and mucin production [244,245]. The only IL-17 antagonist tested in a clinical trial in asthma has been brodalumab, a human anti-IL-17 receptor monoclonal antibody. Busse *et al*., in moderate-severe asthmatics, reported no improvement in lung function or asthma control with 12 weeks treatment with brodalumab, however, pre-specified subgroup analysis showed improvement in asthma control in patients with high FEV_1 reversibility [246].

TNF-α is a pro-inflammatory cytokine secreted by a range of cells including mast cells, eosinophils and macrophages. Airway high TNF-α signalling was demonstrated in patients with refractory asthma compared to both milder asthmatics and non-asthmatic healthy controls [247,248]. TNF-α has been demonstrated to drive vascular remodelling in a murine model of airway inflammation [249]. Anti-TNF-α have an established role in the treatment of chronic inflammatory conditions like rheumatoid arthritis, Crohn's disease and psoriasis. An initial small clinical trial of the anti-TNF-α agent etanercept, a soluble TNF-α receptor, in patients with refractory asthma showed promising results with improvement in FEV_1, AHR and asthma-related quality-of-life scores; however, a subsequent larger RCT using golimumab, a monoclonal antibody against TNF-α, showed no improvement in FEV_1 or exacerbation rate and the trial was discontinued early due to unfavourable risk/benefit profile [248,250]. Unfortunately airway remodelling was never measured as an endpoint in any clinical study involving anti-TNF-α therapy in asthma.

Tyrosine kinase is an enzyme that plays an important role in signal transduction and regulates various cellular activities. Due to their effect on apoptosis, angiogenesis and cellular division, tyrosine kinase inhibitors are used as a biological treatment of various cancers. Signalling of some airway remodelling mediators is through receptor tyrosine kinases which could be blocked by such therapy with direct effect on airway remodelling. EGFR, which is increased in expression in asthma and correlates with

severity, has been linked with epithelial and mesenchymal remodelling changes and could be targeted by tyrosine kinase inhibitors [251-253]. Erlotinib, an EGFR tyrosine kinase inhibitor, in murine asthma models resulted in reduction in ASM mass, RBM thickness, collagen deposition and AHR [251,254]. Stem cell factor is important in mast cells biology and could also be blocked by the tyrosine kinase imatinib. Using another murine model, Rhee *et al.* showed that imatinib reduces ASM thickness [255]. A double blind RCT on the use of imatinib in severe refractory asthma is currently on-going [256]. Masitinib is another tyrosine kinase inhibitor that could potentially be used in the treatment of asthma. In addition to inhibiting stem cell factor receptor (c-kit) it also targets platelet-derived growth factor receptor. In a phase IIa RCT involving 44 patients with severe corticosteroid-dependent asthma, 16 weeks of treatment with masitinib resulted in improvement in asthma control [257]. A phase III multicenter RCT using masitinib in patients with severe persistent asthma who are on maintenance oral GCs is currently recruiting [258].

1.4.2.8. Statins, Metformin and Vitamin D

Statins are inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase enzyme and are used for therapeutically inhibiting cholesterol biosynthesis and reducing cholesterol levels. It has been suggested that statins have a beneficial effect in asthma. However, a systematic review concluded that although statins had an anti-inflammatory effect in asthma, they did not significantly affect lung function or symptoms [259]. On the other hand, a recent retrospective review of obese patients with severe asthma showed that being on statins was associated with better symptom control suggesting a role for statins in this specific asthma phenotype [260]. The effects of stains on inflammation and remodelling have been studied mainly using simvastatin on animal models of allergic asthma. Simvastatin has been shown to inhibit epithelial IL-13-inducable pro-inflammatory cytokines production [261]. Ahmad *et al*. showed that

simvastatin in a murine model resulted in reduction in inflammatory cells infiltration, mucus hypersecreation, epithelial injury, collagen deposition and AHR. This was shown to be medicated by the influence of simvastatin on nitric oxide metabolism [262]. Zeki *et al.*, in a similar model, showed reduction in goblet cell hyperplasia and lung arginase, an important mediator of vascular remodelling in asthma [263]. *In vitro*, simvastatin was shown to inhibit ASM proliferation, which was mediated through RhoA inactivation; and to reduce TGF-β1-induced fibronectin production by human airway fibroblasts [264,265].

Metformin is a biguanide used in the treatment of type 2 diabetes mellitus. It possesses anti-inflammatory and antioxidant properties and has been shown to improve tissue injury and remodelling in models of various diseases [266,267]. In a murine asthma model, through the activation of AMP-activated protein kinase (AMPK), metformin resulted in the attenuation of eosinophilic inflammation, peribronchial fibrosis and ASM hypertrophy. Moreover, the authors also demonstrated, *in vitro*, decreases TGF-β1-induced fibronectin expression in cultured fibroblasts which was also mediated through AMPK activation [268].

Vitamin D and its deficiency have been implicated in airway remodelling in asthma. Gupta *et al.* demonstrated that vitamin D levels are significantly lower in children with severe therapy-resistant asthma and this was associated with increased ASM mass, poor lung function and worse asthma control [269]. *In vitro*, vitamin D has been shown to inhibit platelet-derived growth factor-mediated human ASM cell proliferation through the inhibition of phosphorylation of both checkpoint kinase-1 and the retinoblastoma protein [270]. This negative effect of vitamin D on ASM proliferation could also results from the inhibition of NF-κB signalling which has also been demonstrated on human ASM cells [271].

Table 1.2: Summary of the effects of conventional /experimental asthma therapies/drugs on airway remodelling

Therapy/Drug class	Effect on airway remodelling	Type of evidence/studies
Corticosteroids	Improvement in features of epithelial, mesenchymal and vascular remodelling.	Clinical **(See Table 1.3)**
	Reduction of human ASM proliferation. Inhibition of ASM hyperplasia.	Animal and *in vitro* [168,169,272]
Long acting β2-adrenergic receptor agonists	Enhances the beneficiary effect of GCs on lung fibroblast proteoglycan production when combined with budesonide.	*In vitro* [177,178]
Leukotriene modifiers	Improvement in epithelial desquamation, ASM hyperplasia, mucus plugging and ECM remodelling.	Animal [190-193]
Methylxanthines	Unknown.	-
Cholinergic antagonists	Prevention and reduction of ASM remodelling, mucus gland hypertrophy, Goblet cell hyperplasia and airway fibrosis.	Animal and *in vitro* [203,208,210,211]
Anti-IgE therapy	Reduction of RBM thickness. Improvement of CT-derived morphometry features.	Clinical [221,222]
	Inhibition of ASM proliferation and ECM protein production by ASM cells in culture.	*In vitro*[31]
Interleukin-5 antagonists	Improvement of sub-epithelial fibrosis Improvement of CT-derived morphometry features.	Clinical and animal [9,227,233]
Bronchial Thermoplasty	Reduction of ASM mass	Clinical and animal [273-275]
Anti-IL-13	Possible effect on mesenchymal fibrosis	Clinical [239]
Anti-IL-17	Unknown.	-
Anti-TNF-*alpha*	Unknown.	-

Therapy/Drug class	Effect on airway remodelling	Type of evidence/studies
Tyrosine kinase inhibitors	Reduction of ASM mass, RBM thickness and collagen deposition	Animal [251,254]
Statins	Reduction of mucus hypersecreation, epithelial injury and ECM collagen deposition	Animal [262,263]
	Inhibit ASM proliferation and reduce fibroblast fibronectin production	*In vitro* [264,265]
Metformin	Attenuation of peribronchial fibrosis and ASM hypertrophy	Animal [268]
	Reduction of fibronectin expression in cultured fibroblasts	*In vitro* [268]
Vitamin D	Inhibition of platelet-derived growth factor-mediated human ASM cell proliferation	*In vitro* [270]
PGD_2 antagonists	Unknown	-

Abbreviations: ASM, airway smooth muscle; CT, computed tomography; ECM, extracellular matrix; GCs, glucocorticoids; RBM, reticular basement membrane; TNF, Tumour necrosis factor

Table 1.3: Summary of the clinical studies assessing the effect of glucocorticoid therapy on airway remodelling in asthma

Remodelling feature	Effect	Study	N	Duration	Summary of evidence/study
Mesenchymal fibrosis	**Improve**	Hoshino *et al.* [147]	30	6 months	RCT. Treatment with Inhaled BDP, 800 µg daily, resulted in the reduction of mean RBM thickness from 11.06 µm to 8.34 µm.
		Ward *et al*. [148]	35	12 months	RCT. Treatment with inhaled fluticasone propionate, 1500 µg daily, resulted in reduction of RBM thickness by a mean of 1.9 µm. RBM Changes seen at 12 months, but not at 3 months
		Hoshino *et al*. [153]	24	6 months	RCT. Treatment with Inhaled BDP, 800 µg daily, resulted in mean reduction of RBM thickness from 8.31 µm to 6.07 µm.
		Sont *et al*. [149]	75	24 months	RCT. AHR-guided treatment with high dose ICS resulted in reduction of RBM thickness by a mean of 1.9 µm.
		Chetta *et al*.[150]	16	6 weeks	RCT. High dose inhaled fluticasone propionate, 1000 µg daily resulted in mean reduction of RBM thickness from 9.7 µm to 6.4 µm (n=8). This was not seen with lower ICS dose treatment (n=8)
		Trigg *et al*. [151]	25	4 moths	RCT. Inhaled BDP, 1000 µg daily, resulted in mean reduction of RBM collagen III thickness from 29.7 µm to 19.8 µm.
		Olivieri *et al*. [152]	17	6 weeks	RCT. Inhaled fluticasone propionate, 500 µg daily, resulted in mean reduction of RBM thickness from 14.0 µm to 10.7 µm.
	No effect	Lundgren *et al*. [154]	6	10 years	Severe asthmatics. No significant change in RBM thickness after 10 years of treatment with ICS.
		Jeffery *et al*. [155]	6	4 weeks	Mild asthmatics. Inhaled budesonide, 200 µg daily, resulted in no change in RBM thickness.
		Boulet *et al*.[156]	32	8 weeks	Treatment with inhaled fluticasone propionate, 1000 µg daily, resulted in no change in type I and type III collagen deposition.
		Bergeron et al. [157]	12	6 weeks	No change in collagen deposition after treatment with inhaled flunisolide.

Remodelling feature	Effect	Study	N	Duration	Summary of evidence/study
Epithelial remodelling	**Improve**	Lundgren *et al*. [154]	6	10 years	Severe asthmatics. Reduction in non-ciliated epithelium after 10 years of treatment with ICS
		Laitinen *et al.* [164]	7	3 months	RCT. Inhaled Budesonide,1200 μg daily, resulted in improvement in the number of ciliated epithelial cells
	No effect	None	-	-	-
Vascular remodelling	**Improve**	Chetta *et al*. [150]	16	6 weeks	RCT. High dose inhaled fluticasone propionate, 1000 μg daily, resulted in reduction in the vascular area (n=8). This was not seen with lower ICS dose treatment (n=8).
		Orsida *et al*. [172]	22	-	Cross-sectional study. Asthmatics on ICS dose >/=800 μg /day BDP had reduced number of vessels/mm^2 of lamina propria compared with patients not on ICS and those receiving </=500 μg /day BDP.
		Hoshino *et al*. [173]	28	6 months	RCT. Treatment with Inhaled BDP, 800 μg daily, resulted in the reduction of both vessel number and percent vascularity in the submucosa.
		Feltis *et al*. [82]	35	3 months	RCT. Vessel sprout staining was reduced with inhaled fluticasone propionate, 1000 μg / day, treatment.
	No effect	Orsida *et al* [175]	45	3 months	RCT. Low dose inhaled fluticasone propionate resulted in no improvement in vascularity.
Goblet cell hyperplasia	**-**	None	-	-	-
Increased ASM mass	**-**	None	-	-	-
Mucus gland enlargement	**-**	None	-	-	-

Abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BDP, beclometasone dipropionate; ICS, inhaler corticosteroids; RBM, reticular basement membrane; RCT, randomised controlled trialmembrane; TNF, Tumour necrosis factor

1.5. Prostaglandin D_2 (PGD_2) and asthma

1.5.1. Role of PGD2 in the pathogenesis of eosinophilic asthma

Prostaglandin D_2 (PGD_2) is arguably the most significant prostanoid in the pathogenesis of eosinophilic inflammation in asthma and other atopic disorders. It is primarily produced by mast cells in response to their activation through the TH_2 immune response. Antigens from allergens are presented by antigen-presenting cells to naive T cells which acquire a Th_2 phenotype and start to produce TH_2 cytokines namely IL-4, IL-5 and IL-13. IL-4 and IL-13 stimulate B-lymphocytes to produce IgE. This in turn binds to mast cells leading to their activation and degranulation releasing various inflammatory mediators and cytokines including PGD_2 [24]. Besides affecting the airways directly by causing bronchoconstriction, oedema and increased mucus production; PGD_2 acts on the specific DP_2 receptor, also known as chemoattractant receptor homologous molecule expressed on Th_2 cells ($CRTh_2$), on Th_2 lymphocytes and eosinophils facilitating their chemotaxis and activation, thus playing a central role in activation and propagation of eosinophilic inflammation [25].

PGD_2 has been shown to be increased in the bronchoalveolar lavage of patients with severe asthma and is linked to exacerbations [276]. Stinson *et al.* in a recent report have shown that the number of DP_2+ cells in the bronchial submucosa increases with increasing severity of asthma. Furthermore they have also shown that DP_2 is also expressed by epithelial cells and directly promotes their migration and metaplasia [277].

1.5.2. PGD_2 antagonism in eosinophilic asthma

There are very few clinical studies of using DP_2 antagonists in asthma with variable results. The compound AMG853 was tested in a small RCT of moderate-to-severe asthmatics where it showed no effect on symptoms or lung function. Three RCTs studied the effect of the DP_2 antagonist OC000459 in asthmatics with improvement in lung function and quality of life in the treatment groups [278-280]. The effects

were more pronounced in eosinophilic patients. Another DP_2 antagonist is BI671800. This compound was studied in a RCT with six weeks treatment resulting in some improvement in FEV_1 both in patients on ICS and steroid naïve patients [281]. However, a recent large RCT of BI671800 did not show this improvement [282].

Although the benefits of DP_2 antagonism in the aforementioned studies were only present in some and not all, and only of a modest impact, these studies did not target patients with the eosinophilic phenotype where TH_2 immunity and hence PGD_2 is central in the disease mechanism. Moreover, none of these studies explored the effects of these compounds on airway remodelling.

1.6. Bronchial Thermoplasty

Bronchial thermoplasty (BT) is the only FDA-approved non-pharmacological asthma therapy. It is also claimed to be the only asthma treatment that primarily and directly targets airway remodelling. It is a novel non-drug technique licensed for the treatment of severe persistent asthmatics who do not achieve control despite maximal medical therapy. It employs temperature-controlled radio frequency (RF) energy to target proximal airways, presumably acting on ASM, resulting in clinical benefit.

1.6.1. History of development and mechanism of action of BT

The use of radiofrequency energy to generate heat targeting muscle cells is well established in the medical discipline of cardiology. RF has long been used to treat conduction defects and arrhythmias by targeting specific areas of the cardiac muscle and inducing thermal muscle cell death [283]. The effect of RF on ASM has initially been seen in dogs and in patients with lung cancer treated with the technique. The first proof of concept study was published by Danek *et al.* in 2004 where applying BT to dogs resulted in reduction of ASM layer which was largely replaced by a mature collagen layer [274]. This was accompanied by, and correlated with, significant reduction in AHR.

The first study of BT in humans was published the year after where the treatment was applied to nine patients who were undergoing lung resection for proven or suspected lung cancer. Three weeks before the planned resection, BT was applied only to the segments and lobes to be removed. This was primarily a feasibility and safety study. It showed that BT was safe and resulted in 50% reduction in ASM with the effects of the treatment limited to the treated airways and peribronchial regions [273].

1.6.2. BT studies and trials in asthma

Several studies were done thereafter to assess the clinical benefits of BT in asthmatics. The first was reported by Cox *et al*. who showed that in 16 patients with mild to moderated asthma, BT was safe and resulted in improvement in peak flow measurements, symptom free days and AHR, with the latter persisting up to 2 years post treatment [284]. Following this, a larger RCT study, Research in Severe Asthma (RISA) trial, showed that BT in symptomatic severe asthmatics (n=15) resulted in improvement in asthma control and rescue medication use compared to controls who were treated with standard medical management only (n=17) [285]. A similar but larger RCT was conducted by Cox et al. on uncontrolled asthmatics treated with LABA and ICS, the Asthma Intervention Research (AIR) Trial (n=112). This had shown that BT was associated with improved asthma control questionnaire score (ACQ), asthma quality of life questionnaire score (AQLQ), symptom free days, exacerbation rate, morning peak flow and rescue medication use; however, no difference was found in AHR or FEV_1 compared to control [286]. Finally, the largest, most robust and most recent has been the AIR2 trial. This was a multicentre, randomized, double-blind, sham-controlled clinical trial involving 288 patients (treatment to sham ratio of 2:1) [287]. The primary outcome of this trial was the difference in AQLQ from baseline to average of 6, 9 and 12 months (integrated AQLQ) in subjects receiving BT compared to sham. BT was associated with superior improvement in AQLQ compared to sham (1.35 $\pm$ 1.10 BT; 1.16 $\pm$ 1.2 sham). The proportion of patients with AQLQ improvement of more than 0.5 (minimal important difference)was

significantly higher in the BT group (79%) compared to sham (64%). Moreover, compared to sham, the BT group had significant reduction in severe exacerbations (0.48 vs. 0.7 exacerbation/subject/year), emergency department (ED) visits (0.07 vs. 0.43 [ED visit]/subject/year) and days missed from work/school/other activities due to asthma (1.3 vs. 3.9 days). This amounted to 32% reduction in the rate of severe exacerbations and 84% risk reduction in asthma-related ED visits flowing BT. However, there was no significant differences between the two groups in asthma control scores, rescue medication use, airflow obstruction measures or symptom-free days. Most of the patients in the BT group were followed up in the second year following treatment revealing same rate of exacerbations, adverse events, and hospitalization as the first year [288]. Reduced rates of both severe exacerbations and ED visits for respiratory symptoms were further demonstrated in 85.3% of the BT group patient who were followed up annually for 5 years [289].

1.6.3. Limitation of the current evidence supporting BT use in severe asthma

The AIR2 trial and its follow up studies have significant shortcomings that are difficult to ignore [290]. Although this study was sham controlled, most patients in the BT group following their second treatment session could correctly guess their group allocation [287]. This obviously introduces the possibility of observer bias. Another weakness of the study is the exclusion of severe asthma patient with any of the following 1) frequent hospital admissions or lower respiratory tract infection (≥ 3 times), 2) FEV1 <60% predicted, 3) frequent exacerbations, and 4) patients on regular oral prednisolone dose of ≥10 mg/day. This excludes a large proportion of patients who fit the agreed definition of severe or refractory asthma and even have more severe disease compared to the patients included in the study. It also puts into question the use of both exacerbation frequency and hospital admission rate as a valid outcome measure.

Critically, the primary endpoint of improvement in the total AQLQ score was not actually significantly different between the two treatment groups. Patients in the

treatment group improved by 1.35 ± 1.10 while patients in the sham group also improved by 1.16 ± 1.23. This difference in the magnitude of AQLQ improvement of 0.19 was not statistically or clinically significant, as it is below the target posterior probability of superiority 96.6%, and below the agreed minimal clinically relevant threshold of 0.5 [291]. Although, as earlier mentioned, further analysis by the authors of AIR2 showed that a statistically larger proportion of the BT group achieved an improvement of total AQLQ > 0.5; this was not *a priori* endpoint. Furthermore, none of the secondary outcome measures (ACQ, rescue medication use, FEV_1 and peak flow measurements) were significantly different between the two groups [287]. Indeed all the other outcome measures which favoured BT over sham were designed as safety measures and were not a significant factor when planning the study's statistical analysis.

Although two AIR2 follow up studies suggested continued benefit from BT up to 5 years following the treatment, these did not include the sham control patients and are thus difficult to interpret with certainty [288,289]. Interestingly, a recent systematic review on BT by The Cochrane Collaboration, which included 3 BT clinical trials (AIR, RISA and AIR2), concluded that BT for asthmatics with moderate to severe disease imparts modest clinical benefits in quality of life and exacerbations rates [292]. One weakness of this report is that it included two studies that unblinded and had no appropriate comparable control group (BT was compared to standard therapy).

1.6.4. BT mechanisms of action: the unanswered questions

The exact effects of BT on the airway structure and how that leads to clinical befits are not fully known. The initial evidence for the effect of BT on remodelling is from healthy dogs and cancer patients undergoing resection where it was shown to reduce ASM mass. A very recent case series has shown that BT reduced ASM mass in 10 severe asthmatics 3 months following treatment [293]. In this report mean ASM% dropped from 20% to 7% following BT, however this study has important limitations.

One critical limitation is that the authors only selected patients with ASM% >15% to be included in the study (from personal communications with the authors), which obviously introduces a high risk of regression bias. The evidence of such bias is supported by the finding of reduced ASM% in biopsies taken from the middle lobe in 7 out of the 10 patients (relative average reduction of 48%) [293]. Due to its inherent risk of collapse, the middle lobe is not normally treated during BT, thus samples from middle lobe were initially planned to be used as control. The authors explain the effect of BT on the middle lobe by the possible diffusion of heat from the treatment of close-by airways, however this is not backed by any solid evidence apart for the finding of peribronchial pneumonitis on a CT scan of one patient showing middle lobe changes 1 day following the third BT session where both upper lobes were treated. However, this CT change is unlikely to be resulting from the direct effect of heat, as anatomically the sampled middle lobe bronchi are significantly distant from any treatable airway (>3mm in diameter) in the right upper lobe, and more likely to represent obstructive pneumonitis from excessive secretions known to happen during BT.

The inconsistent effect of BT on AHR and the absence of spirometry improvement are at least curious and raise important questions regarding the mechanism of action underpinning the apparent benefits seen following BT. It has been suggested that BT might be ablating a form of ASM pacemaker in the proximal airways with effect propagating distally, although this is no more than a hypothesis [294]. Examples of alternative mechanisms of action of BT include influencing mucus production or altering other mesenchymal cells behaviour are possible but need to be assessed by future studies. Interestingly, a recently reported case series showed reduction in ASM, although the number of participants was too small (3) to make any informative conclusions [295].

1.7. Hypothesis and aims

1.7.1. Central hypothesis

I hypothesise that, in asthma, airway structural remodelling:

1. Is a significant determinant of abnormal airway physiology,
2. Could be assessed non-invasively by quantitative computed tomography,
3. Could be targeted and modified by the DP_2 antagonism; and
4. Is altered by bronchial thermoplasty by the actions of thermoplasty on airway tissue structures other than airway smooth muscle cells.

1.7.2. Aims

- To demonstrate that proximal airway remodelling as assessed by the microscopic examination of endobronchial biopsies changes is an important predictor of airflow obstruction in moderate-severe asthmatics.

- To assess the relationship between proximal airways microscopic remodelling and airway morphometry as measures by computed tomography.

- To investigate the effect of 12 weeks therapy with fevipiprant, a PGD_2 antagonist, on airway inflammation and airway remodelling in eosinophilic asthma.

- To investigate the effect of bronchial thermal therapy on airway remodelling as assessed microscopically by using histological and immunohistochemical methods, and radiologically by using qualitative computed tomography

2. CHAPTER TWO: MATERIALS AND METHODS

2.1. Clinical Methods

2.1.1. Spirometry

Spirometry was performed as per the American Thoracic Society / European Respiratory Society (ATS/ERS) guidelines by trained operators [296]. The Compact Vitalograph spirometer (Vitalograph, Buckinghamshire, UK) machine was used. Calibration and quality control of all equipments was done in accordance to the aforementioned guidelines. To perform a test procedure, subjects were asked to perform a forced vital capacity (FVC) manoeuvre using the open circuit method. After ensuring correct posture and clear understanding of the instructions, subjects, from a status of functional residual capacity (FRC), were asked to inhale rapidly to total lung capacity (TLC). With a pause of less than 1 second at TLC the subject then places the his lips around the mouthpiece ensuring good seal then exhales rapidly and completely until no more air can be exhaled. A minimum of 3 acceptable FVC manoeuvres were performed and the test was only deemed acceptable if the difference between two largest recordings of FVC and forced expiratory volume in 1 second (FEV_1) were <150 ml. If this was not met then more FVC manoeuvres were performed until the criteria were met or up to a maximum of 8 times. The largest values of both FEV_1 and FVC were recorded as the final result. The European Coal and Steel Community regression equations were used to determine predicted values [297].

Bronchodilator reversibility was done by repeating spirometry 15 minutes after the administration of 400 micrograms of salbutamol via a spacer device.

2.1.2. Sputum induction

Sputum induction and analysis was performed as previously described [298]. Briefly the patient was given 200 micrograms of inhaled salbutamol prior to staring sputum

induction. Au ultrasonic nebuliser was use to sequentially nebulise 3%, 4% and 5% saline for 5 minutes each. The patient is instructed to tidal breath throughout the nebulisation. After rinsing their mouth and blowing their nose, the subjects were instructed to expectorate sputum into a sterile pot. Due to the risk of hypertonic saline causing bronchoconstriction FEV_1 was monitored after each nebulisation. If the FEV_1 dropped by more than 20% then the procedure was stopped. If it dropped by <20% but >10% then the concentration of the saline was not changed for the subsequent nebulisation round.

2.1.3. Bronchoscopy and endobronchial sampling

Fibre-optic bronchoscopy was performed according to the British Thoracic society guidelines [299]. All patients received 2.5mg Salbutamol nebuliser prior to the procedure. All patients were offered intravenous midazolam for sedation. Endobronchial biopsies were obtained from segmental and subsegmental carina and either embedded in plastic resin (glycol methacrylate) or paraffin wax.

2.1.4. Measurement of health-related quality of life & Asthma Control

2.1.4.1. Juniper Asthma Quality of Life Questionnaire (AQLQ)

The Asthma Quality of Life Questionnaire (AQLQ) is a validated, self-administered, disease-specific, paper based tool used to measure asthma health-related quality of life [300]. The questionnaire is composed of a total of 32 items which cover 4 functional domains: Activity Limitation (12 items), Emotional Function (5 items), and Environmental Exposure (4 items). The standardised version of the AQLQ was used. The score for each question is given in a 7 point scale (higher score indicating better quality of life). Final AQLQ scores are calculated for each domain (as the arithmetic mean of specific domain items scores). An overall AQLQ score was also obtained as the mean of all 32 items scores. As previously validated, a value of 0.5 was considered as the minimally important difference [291].

2.1.4.2. Juniper Asthma Control Questionnaire (ACQ)

The Asthma Control Questionnaire (ACQ) is simple validated tool for quantifying asthma control with a recall period of 1 week [301]. It is composed of 7 items; the first 5 are self-administered and assess night and daytime symptoms and activity limitation. Item 6 evaluates short acting bronchodilator use while 7 is concerned with the degree of airflow limitation (FEV_1 %predicted).The responses for all the items are on a 7-point scale ranging from 0 (no limitation) to 6 (severe limitation). Two final ACQ scores were expressed; ACQ6 which is calculated by obtaining the mean value for the scores of the first 6 items only (excluding item 7), and ACQ7 which included all the items. A value of 0.5 was considered the minimum clinically important change. A cut-off of 1.5 was used as to define adequate (<1.5) and poor (≥1.5) asthma control [302].

2.2. Laboratory techniques and methods

2.2.1. Sputum processing and analysis

After the removal of contaminating saliva, the sputum was weighed and added to 0.1% dithiothrietol (DTT). The volume of DTT added was calculated as 4x volume / weight of sputum. The sputum was then put through a vortex device for 15 seconds followed by a bench spiromix for 15 minutes. An equal volume of Dulbecco's phosphate buffered saline (D-PBS) was added before filtering though a nylon gauze and then centrifuged 2000rpm for 10 minutes. After removal of the sputum supernatant, the cells were suspended in PBS and then cell viability was assessed using haemocytometer counting chamber after mixing the suspension with tryptan blue. PBS was then used to make the suspension cell count 0.5-0.75 x 106 cells/ml. The resulting suspension then underwent cytospin centrifugation at 450rpm (18.1g) for 6 minutes. The cytospins were then stained and fixed using Romanowski stain. Differential cell counts were recorded by a single blinded individual and expressed as percentage values of a sample containing at least 400 non-squamous cells [298].

2.2.2. Endobronchial biopsies processing

2.2.2.1. Fixation and embedding

Depending on the study, biopsies were either embedded in plastic resin or paraffin wax. After endobronchial sampling at bronchoscopy, for plastic resin embedding, biopsies were immediately transferred to ice cold acetone solution containing phenylmethane sulfonyl fluoride (PMSF), a serine protease inhibitor, for fixation. After storage for 24 hours at -20°C the fixative is replaced, at room temperature, by acetone for 15 minutes and then methyl benzoatefor another 15 minutes. The biopsies are then infiltrated by glycol Methacrylate (GMA) by putting them in 5% methyl benzoate in GMA 4°C for 2 hours. This is repeated three times. The biopsies are then embedded in GMA which polymerises overnight using a catalyst at 4°C. The blocks are then stored at -20°C.

For conventional paraffin wax embedding, biopsies were fixated in 10% neutral buffered formalin and then embedded in paraffin wax and stored at room temperature [303].

2.2.2.2. Section preparation

Sections were cut at 2 micrometers and then immediately floated in a water bath containing ammonia (0.2%) for about 1 minute. Ammonia helps with antigen expression and makes staining easier. The sections were picked up on slides and were ready for staining after 4 hours drying period. For Haematoxylin and eosin (H&E) staining, the slides were stained with Haematoxylin (Pioneer Research Chemicals), then washed and then stained with eosin before being washed, dried and covered. For Periodic Acid Schiff (PAS) staining the slides were oxidised in 0.5% periodic acid solution for 5 minutes then rinsed before being placed in Schiff reagent for 15 minutes. The slides were then washed and stained with haematoxylin.

For immunohistological staining the EnVision FLEX™ immunohistochemical kit (Dako) was used as per manufacturer's instructions. In brief, the slides were subjected to

EnVision™ FLEX Peroxidase-Blocking Reagent (RTU) then washed with the supplied buffer before applying the primary antibodies and washed with buffer again. EnVision™ rabbit linker was then added and washed before adding EnVision™ FLEX /HRP (RTU) for 20 minutes and washed. The slides were then optimised using Envision™ FLEX Substrate Working Solution before finally staining with haematoxylin.

2.2.3. Biopsy analysis

For image analysis and morphometry ZEN 2012 image analysis software for light microscopy (Carl Zeiss AG, Jena, Germany) was used. All morphometry measurements and cellular counts were performed by one observer on two non-contiguous tissue sections at least 20µm apart from the same biopsy block. The final areas and counts were the average of the measurements from the two sections. Inter- and intra-observer reproducibility of most remodelling markers was assessed in 11 validation subjects. See table 2.1.

2.2.3.1. Tissue areas measurements

Tissue areas were measured in Haematoxylin & Eosin (H&E) or smooth muscle actin (anti-human smooth muscle actin (SMA), clone 1A4, Dako UK, Ely, United Kingdom) stained slides. Total area, airway smooth muscle area and epithelial area were measures directly, while the lamina propria (LP) area was calculated by subtracting all the other areas and the area occupied by vessels and lymphatics from the total section area (LP area= total area – (ASM + epithelium + gland + damaged area + vessel area). All areas were expressed in mm^2 and also as percentages of the total identified areas (e.g. ASM% = ASM X 100/ (ASM + epithelium + gland + LP)).

For all tissue areas, the intraclass correlation coefficient (ICC) for intra-observer repeatability, staining method variability (2day method vs. EnVision method), and variability of measurements in H&E compared to SMA stained slides, was almost perfect.

2.2.3.2. Reticular basement membrane (RBM) measurement

Subepithelial fibrosis was quantified by measuring the thickness of the RBM (a commonly used misnomer of the lamina reticularis layer) as previously validated by Sullivan *et al* [113]. RBM thickness was measured at x200 magnification by measuring 50 points 20µm apart. Only tangentially orientated RBM was measured with each measurement line was drawn perpendicular to the tangent of the epithelial (outer) border of the RBM. The final RBM thickness was calculated as the arithmetic mean of all 50 measurements.

2.2.3.3. Assessment of goblet cells numbers

Epithelial goblet cells and their mucus content was assessed on periodic acid-Schiff (PAS) and MUC5AC (Anti-mucin 5AC, clone 45M1, Abcam, Cambridge, UK) stained sections. At x200 magnification, areas of intact and tangentially orientated epithelium were identified and both the area and the lengths of the outer border of the corresponding RBM were measured. Goblet cells within those epithelial areas were then enumerated and the final goblet cells numbers were expressed, using both stains, as goblet cells/mm^2 of intact epithelium area and /mm of corresponding RBM.

2.2.3.4. Thresholding for MUC5AC and actin expression

Mucus goblet cells content of intact epithelium on MUC5AC stained slides and acting staining intensity on α-SMA stained slides were assessed using a thresholding technique based on the hue, saturation, and intensity (HSI) method for colour detection [304]. The HSI colour scale ranged from 0 to 360 for hue and 0 to 255 for both saturation and intensity. Images were acquired at ×100 magnification following correction for white balance. The final ideal threshold for MUC5AC detection was determined by selecting the median of all the lower and upper limits of the HSI used in 12 validation patients with different MUC5AC staining intensity and mucus content. Similar validation was done for α-SMA. After validation, the following HIS setting was selected and applied for thresholding of all the MUC5AC biopsies slides:

hue (upper limit, 345; lower limit, 44), saturation (upper limit, 255; lower limit, 6), and intensity (upper limit, 207; lower limit, 22). For α-SMA thresholding the following HSI setting was applied: hue (upper limit, 300; lower limit, 7), saturation (upper limit, 255; lower limit, 10), and intensity (upper limit, 30; lower limit, 8). The quantification of mucus content and MUC5AC staining was expressed as the percentage of the intact epithelium occupied by MUC5AC staining (i.e. above the predetermined threshold). The quantification of α-SMA expression was assessed as percentage of both ASM and total section areas occupied by α-SMA stained areas which are deemed above the predetermined threshold.

2.2.3.5. Epithelial thickness

Epithelial thickness was measured at x200 magnification by using the method described by Cohen *et al.* [305]. Areas of intact and tangentially orientated epithelium were identified and measured. Subsequently, to calculate the epithelial thickness, this area was divided by the lengths of the outer border of the corresponding RBM. The epithelial thickness was expressed in $\mu m^2/\mu m$. In 11 subjects the ICC for intra-observer variability was 0.87.

2.2.3.6. Epithelial integrity

Epithelial integrity was assesses by measuring the lengths of intact epithelium, denuded epithelium and partial epithelium (not intact and not denuded). These were expressed as percentage of all the RBM length present in the section.

2.2.3.7. Assessment of vascular remodelling

Vascularity and vascular remodelling was quantified on slides stained with anti-endothelium clone EN4 antibodies (Monosan, Uden, The Netherlands). This was done by measuring the mean Chalkley count, a surrogate vascular marker that expresses both vessel number and vascular area. As previously described, a Chalkley eyepiece graticule (NG52 Chalkley Point Array, Pyser-SGI Ltd, Edenbridge, UK), composed of a circle with 25 randomly positioned dots, was used, at x200, in four

non-overlapping vascular hotspots (1-2/section) [81]. On each hotspot the graticule was rotated and stage was moved so the maximum numbers of dots are within or touching EN4+ vessels. The dots are counted for each hot spot and the mean Chalkley count is the mean of the 4 counts. See Figure 3.8.

2.3. Radiological methods

Subjects underwent either limited or full lung CT scans using a Siemens CT scanner (Siemens Sensation 16; Siemens, Surrey, United Kingdom). CT scans were performed within 60 minutes of the administration of 400 mcg inhaled salbutamol via a spacer device (AeroChamber® Plus). Scans were acquired in the supine position with the shoulders fully abducted to avoid bone artefact. For image analysis, a fully automated CT analysis software, the Volumetric Information Display and Analysis (VIDA) Pulmonary Workstation, version 2.0 (PW2 software; VIDA Diagnostics, Coralville, Iowa) was used for quantitative airway morphometry (limited and full lung scans) and lung densitometry (full scans only). Images were acquired at collimation of 16 × 0.75 mm, pitch of 1.5 mm, 120 kVp, 40 mA and 0.5-second rotation time. Images were reconstructed with a slice thickness of 0.75 mm and a slice interval of 0.5 mm by using a low spatial frequency algorithm (B35f kernel) through a 512 × 512 matrix.

2.3.1. Full lung CT scans

Subjects underwent full lung inspiratory and expiratory scans. Inspiratory images were acquired at full inspiration (near TLC) while expiratory scans were acquired at the end of expiration (near FRC). Densitometry was calibrated by using density measures of air, blood, and electron density rods which were encased in a foam box (LD15; Styrotech Ltd, West Bromwich, United Kingdom) attached to each subject's anterior chest during scanning. Three electron density rods were in each box (LN300, LN450, and ''solid water'' with an electron density relative to water of 0.28, 0.40, and 0.99, respectively) from an RMI467 electron density CT phantom (Gammex-RMI Ltd, Nottingham, United Kingdom).

2.3.2. Limited CT scans

Limited only scans were done in 14 asthmatics and were analysed retrospectively. All scans were done at full inspiration (near TLC). The subjects had limited scans from the aortic arch to the carina. This was done to capture the mean right upper lobe epical segmental bronchus (RB1) only and was not used in calculating mean segmental bronchus or densitometry calculations.

2.3.3. Quantitative bronchial morphometry

All scans were analysed for RB1 morphometry while mean segmental bronchi morphometry was obtained in full lung scans only. 1^{st}-5^{th} generation airways were labelled and measured using the analysis software. Morphologic measurements of segmental airways, including RB1, were obtained along each centerline voxel of the lumen perpendicular to the long axis on each airway and averaged over the middle third of the airway segment. Lumen area (LA) and total area (TA) were measured directly while wall area (WA) was the derived using the following calculation: WA = TA - LA. TA, LA and WA were measured in mm^2 and all corrected for body surface area (BSA) which was calculated using Mosteller formula and expressed as mm^2/m^2 BSA [306]. Percentage wall area (WA%) was calculated as follows: WA% = 100 x WA/TA.

2.3.4. QCT-derived measures of air trapping

Densitometry measurements were performed on all inspiratory and expiratory full lung scans and were calibrated by using density measures of air, blood, and the electron density rods. Based on the density mask technique, the software is able to delineate the lung from other thoracic structures and generate histogram curves of the lung voxels. Three air-trapping measurements were calculated: (1) MLD E/I. This is defined as the mean lung density on inspiratory scans (at FRC)/mean lung density on inspiratory scans (at TLC), (2) VI-856HU. This is defined as the percentage of lung voxels with a density lower than -856 HU, and (3) CT-TLV E/I. This is defined as CT-

measured total lung volume during expiration / CT-measured total lung volume during inspiration. This is expressed as a percentage.

2.3.5. CT radiation exposure and safety

Patients' radiation exposure caused by research CT scans was assessed using dosimetry calculations. The effective dose for both limited and full lung scans were calculated using the ImPACT CT dosimetry calculator (ImPACT, St. George's Healthcare NHS Trust, London, United Kingdom). The effective radiation dose was calculated based on dose distribution data obtained using the Monte Carlo simulation based on the programme mathematical adult hermaphrodite phantom. The calculated effective dose for all research full and limited scans was ≤ 10 mSv.

2.4. Statistical methods

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com), IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Released 2013, Armonk, NY), and SAS/STAT software, versions 9·3 and 9·4 of the SAS System for AIX (SAS Institute Inc., Cary, NC, USA). Normality assumption was assessed both graphically by generating Q-Q plots of residuals and histograms and statistically by applying the Kolmogorov-Smirnov test of normality. Normally distributed data is expressed as mean (standard deviation, SD or standard error of the mean, SEM) and non-normally distributed data was expressed as median [inter-quartile range, IQR]. Depending on the shape of the histogram distribution, some non-normally distributed data was Log transformed data was presented as geometric mean (95% confidence interval). Linear correlation between variables was measured using the Pearson product-moment correlation coefficient (r) or Spearman rank correlation (r_s), as per distribution. Also depending on normal distribution, student t-test or Mann-Whitney U test was used to compare paired and unpaired groups, while one-way analysis of variance (ANOVA) or Kruskal-Wallis test were used to compare variables in 3 or more groups. Proportions were compared using chi-squared test (χ^2). A step-wise multiple regression analysis was

used to further evaluate the relationship between specific normally distributed independent and dependent variables.

For the randomised controlled trial of fevipiprant (Chapter Four), changes in efficacy outcomes from the baseline to post-treatment visits were analysed using an analysis of covariance (ANCOVA) model, with treatment as the fixed effect. Randomisation strata and baseline values of efficacy variables were entered as factors in the ANCOVA model for analysis of the primary outcome, secondary outcome and exploratory outcomes. Intention to treat analysis was used for the efficacy outcomes while received treatment analysis was used for safety outcomes.

A two-way mixed, single measures (Absolute agreement) model intraclass correlation coefficients, ICC (model 3,1), was used to assess 1) single-measure repeatability of the immunohistological variables measured by a single observer at least 4 weeks apart, 2) inter-observer variability, 3) variability of various immunohistological variables when measured using the 2 day staining technique compared to nVision , and 4) variability of tissue area measurements using H&E compared to SMA stained sections. The following agreement scale for ICC values was adopted: 0.21–0.40 = fair, 0.40–0.60 = moderate, 0.61–0.80 = substantial and 0.81–1 = almost perfect [307].

For all statistical tests and methods, a *p*-value of <0.05 was considered statistically significant.

Table 2.1: Repeatability of airway remodelling markers on biopsies

Remodelling measure	**Intra-observer variability intraclass correlation coefficient**	**Inter-observer variability intraclass correlation coefficient**
Reticular Basement Membrane	0.90	0.85
Vascularity (mean Chalkley count)	0.85	0.65
Tissue areas	0.94	0.81
Epithelial Integrity	0.95	0.84
Epithelial Thickness	0.87	0.69

A two-way mixed, single measures (Absolute agreement) model intraclass correlation coefficients, ICC (model 3,1), was used for both inter- and intra-observer variability. The following agreement scale for ICC values was adopted: 0.21–0.40 = fair, 0.40–0.60 = moderate, 0.61–0.80 = substantial and 0.81–1 = almost perfect.

3. CHAPTER THREE: ASSOCIATIONS IN ASTHMA BETWEEN PHYSIOLOGICAL MARKERS OF AIRFLOW OBSTRUCTION, QUANTITATIVE COMPUTED TOMOGRAPHY AND BRONCHIAL BIOPSY-DERIVED AIRWAY REMODELLING

3.1. Abstract

Introduction: Airway remodelling in asthma remains poorly understood. This study aimed to determine the association of airway remodelling measured on bronchial biopsies with i) lung function impairment and ii) quantitative thoracic CT (QCT)-derived morphometry and densitometry measures of proximal airway remodelling and air trapping .

Methods: Subjects were recruited from a single centre. Bronchial biopsy remodelling features that were the strongest predictors of lung function impairment and QCT-derived proximal airway morphometry and air-trapping markers were determined by step-wise multiple regression. The best predictor of air trapping was validated in an independent replication group.

Results: Airway smooth muscle percentage (ASM %) was the only predictor of post-bronchodilator FEV_1 % predicted, while both ASM % and vascularity were predictors of FEV_1/FVC. Epithelial thickness and ASM % were predictors of mean segmental bronchial luminal area (R^2=0.12; p=0.02 and R^2=0.12; p=0.015). Whereas epithelial thickness was the only predictor of % wall area (R^2=0.13; p=0.018). Vascularity was the only significant predictor of air trapping (R^2=0.24; p=0.001), which was validated in the replication group (R^2=0.19; p=0.031).

Conclusion: In asthma, airway smooth muscle content and vascularity were both associated with airflow obstruction. QCT-derived proximal airway morphometry was most strongly associated with epithelial thickness and airway smooth muscle content, whereas air-trapping was related to vascularity.

3.2. Introduction

Asthma remains an important health problem with significant morbidity, mortality and economic burden [308,309]. In addition to symptoms, asthma is characterized by variable airflow obstruction, airway inflammation and airway remodelling [5,309]. Airway remodelling is a collective term for the structural changes in the airway wall including epithelial thickness and integrity, airway smooth muscle mass, neoangiogenesis and subepithelial fibrosis [5,22,309] and is related to persistent airflow limitation and airflow obstruction [22,41,81]. It is a feature even in childhood asthma [310] demonstrating that it can occur early in disease and post-mortem studies of asthma deaths demonstrate airway remodelling in the large and small airways [311,312].

Macroscopic airway remodelling can be assessed non-invasively by quantitative computed tomography (QCT). This has become an established technique to determine airway morphometry and lung densitometry in asthma [74,127,128,134,227,234,313-316]. This approach allows for quantification of proximal airway remodelling by assessment of airway geometry and air-trapping as an indirect measure of small airway disease. QCT in asthma has revealed that the key features of airway remodelling including luminal narrowing, wall thickening and moreover air-trapping are important determinants of airflow obstruction. Some studies have begun to explore the associations between proximal airway geometry and histological features of airway remodelling [74,127,134]. However, asthma is a heterogeneous condition with considerable variability in the degree of disordered airway physiology, and the relative changes in airway wall composition and QCT parameters. Thus, these structure-function relationships in asthma remain poorly understood.

My hypothesis was that airway remodelling determined in bronchial biopsies is associated with i) lung function impairment (post-bronchodilator FEV_1 % predicted) and ii) QCT morphometry and densitometry measures of proximal airway

remodelling and air trapping. The co-primary QCT outcome variables were: - (i) for proximal airway remodelling: mean airway lumen area / body surface area and wall area % and; (ii) for air trapping: mean lung density expiratory to inspiratory ratio. To test our hypothesis we undertook a single centre observational study across the spectrum of disease severity to determine the strongest independent histological features in bronchial biopsies associated with lung function and QCT parameters of airway remodelling. The best immunohistological predictor of air trapping was validated in an independent replication group of asthmatics from a second centre.

3.3. Methods

3.3.1. Subjects

Subjects were recruited into either test (n=70) or replication (n=24) groups at two independent centres Glenfield Hospital, Leicester, UK and Washington University School of Medicine, St Louis, MO, USA respectively. All subjects were non smokers with <10 pack-years. All included subjects fulfilled the criteria for the diagnosis of asthma which was defined as: a physician diagnosis of asthma with objective evidence of variable airflow obstruction as indicated by 1 or more of the following: (1) a positive methacholine challenge test defined as a concentration of nebulised methacholine causing a 20% drop in FEV_1 of <8 mg/mL, (2) diurnal maximum peak flow variability of >20% over 2 week time, and (3) improvement of >15% in FEV_1 15 minutes after bronchodilator therapy. Subjects underwent pre- and post-bronchodilator spirometry (Salbutamol 400mcg), skin prick tests or allergen specific IgE to assess for atopy and those in the test group also underwent sputum induction and processing for cell count. Persistent airflow limitation was defined as a post-bronchodilator therapy FEV_1<80% predicted. Written informed consent was obtained from all the participants. All subjects were on optimal asthma treatment and free of exacerbation for at least 6 weeks prior to recruitment into the study. All the assessments and tests included in this study were approved by the local research ethics committee (The Leicestershire, Northamptonshire, and Rutland Research

Ethics Committee and the Washington University School of Medicine Institutional Review Board).

3.3.2. Computed tomography

Subjects underwent either limited or full lung CT scans performed following administration of salbutamol using standardised acquisition protocols as described previously [316]. Limited only scans from the aortic arch to the carina done at full inspiration (near total lung capacity) were undertaken in 14 asthmatics [128,234]. All other subjects had full lung inspiratory and expiratory (near functional residual capacity) CT scans. Scans were analysed using semi-automated software, Apollo in the test group and Pulmonary Workstation, version 2.0 in the replication group (VIDA Diagnostics, Iowa). In the test group all inspiratory scans were analysed for the right upper lobe apical segmental bronchus (RB1) morphometry while mean segmental bronchi morphometry was obtained in full lung scans only. 1^{st}-5^{th} generation airways were labelled and measured using the analysis software. Morphologic measurements of segmental airways (3^{rd} generation airways) were obtained along each centre line voxel of the lumen perpendicular to the long axis on each airway and averaged over the middle third of the airway segment. Lumen area and total area were measured directly while wall area was the derived using the following calculation: wall area = total area - lumen area. Total, lumen and wall area were measured in mm^2 and all corrected for body surface area, which was calculated using Mosteller formula and expressed as mm^2/m^2 body surface area [306]. Percentage wall area was calculated as follows: wall area % = 100 x wall area/total area. Estimates of air-trapping were determined in the test and replication groups from the mean lung density on the expiratory/inspiratory scan (MLD E/I) and the percentage of lung voxels with a density lower than -856 HU on expiratory scans (VI-856 HU). (See figure 3.7). The co-primary QCT outcome variables were for proximal airway remodelling: mean segmental airway lumen area / body surface area and wall area % and for air trapping MLD E/I.

3.3.3. Endobronchial biopsies

Fiberoptic bronchoscopy was performed according to the British Thoracic society guidelines [299]. All patients received salbutamol prior to the procedure. Endobronchial biopsies were obtained from segmental and subsegmental carina and either embedded in glycol methacrylate for the test group or paraffin in the replication group as described previously [81,127,304,317].

Two micrometre sections were cut from the glycol methacrylate embedded biopsies and stained with Haematoxylin & Eosin (H&E). Immunohistochemical staining was done with the following mAbs: anti–mast cell tryptase clone AA1 (Dako, UK), anti-alpha smooth muscle actin clone 1A4 (Dako UK), anti-eosinophil major basic protein clone BMK-13 (Monosan, Netherlands), anti-neutrophil elastase clone NP57 (Dako UK), anti-endothelium clone EN4 (Monosan, Netherlands) and anti-Mucin 5AC (MUC5AC clone 45M1 (Abcam, Cambridge, UK) or appropriate isotype controls.

The endobronchial biopsies were assessed by a single observer blinded to the clinical characteristics (ZEN 2012 image analysis software for light microscopy, Carl Zeiss AG, Germany) and expressed as the mean of measurements undertaken from a minimum of two sections either from independent biopsies or as non-contiguous tissue sections at least 20µm apart from the same biopsy. Epithelial integrity was assessed by measuring the lengths of intact and denuded epithelium. These were expressed as percentage of all the reticular basement membrane (RBM) length present in the section. RBM and epithelial thickness were measured as described previously [113,305]. Vascularity was measured using the Chalkley count, a surrogate of both vessel density and vascular area. As previously described, a Chalkley eyepiece graticule (NG52 Chalkley Point Array, Pyser-SGI Ltd, UK) was used at x200 to measure Chalkley counts in four non-overlapping vascular hotspots (1-2/section) (See Figure 3.8)[81]. The mean Chalkley count was calculated from the four measurements. In 24 biopsies we compared the Chalkley count with computerised pixel counting in the lamina propria and found these measures were strongly correlated (r=0.83;

p<0.0001). Airway smooth muscle content was determined as the proportion of the total area. Inflammatory cells were expressed as the number of nucleated cells/area of lamina propria. Goblet cell were enumerated on MUC5AC stained slides and expressed as goblet cells / mm^2 of intact epithelium

The intra-class correlation coefficients (ICC) for the within donor measurements made by a single-blinded observer were for airway smooth muscle % area (ICC=0.87), epithelial thickness (ICC=0.85) and vascularity (ICC=0.65).

The strongest independent immunohistological feature of airway remodelling associated with QCT-derived air trapping identified in the test group was validated in the replication group. Four micrometre sections were cut from the paraffin embedded biopsies and stained with appropriate mAb or corresponding isotype control.

3.3.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California) and IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Released 2013, Armonk, NY). Parametric data were expressed as mean (standard deviation, SD) and non-parametric data as median (interquartile range, IQR). Groups were compared using unpaired student t-test and Mann-Whitney U test for parametric and non-parametric data respectively. Proportions were compared using chi-squared test (χ^2). Correlations between variables were expressed using Pearson's correlation. A step-wise multiple regression analysis was undertaken to determine the bronchial biopsy features that were the strongest predictors of post-bronchodilator FEV_1, FEV_1/FVC, and QCT-derived mean segmental bronchial morphometry and air-trapping. Regression data are presented as model-adjusted R^2 Pearson correlations alongside the standardized regression coefficient (β) of the modelled independent variable. A *p*-value of <0.05 was considered statistically significant.

3.4. Results

Baseline demographics and clinical characteristics of subjects with (n=30) and without persistent airflow limitation (post-bronchodilator FEV_1 <80% and ≥80% predicted) (n=40) are shown in Table 3.1. There was no difference between the two groups in sex, age, duration of asthma, age of disease onset, smoking status, smoking pack-years, BMI, sputum eosinophils or sputum neutrophils.

3.4.1. Airway inflammation and remodelling univariate correlation with lung function

Subjects with versus those without persistent airflow limitation had significantly higher airway smooth muscle % (33.5 [15.6] versus 20.1 [12.6]%; p<0.001) and increased vascularity (mean Chalkley count) (6.2 [1.6] versus 5.0 [1.9]; p=0.017) as shown in Table 3.1 and Figure 3.1. However, there was no difference between the two groups in the other measured markers of airway remodelling or inflammation. Airway smooth muscle % was inversely correlated with post-bronchodilator FEV_1 % predicted (r=-0.49; p<0.001) and post-bronchodilator FEV_1/FVC (r=-0.44; p<0.001) as shown in Figure 3.2. Vascularity was also inversely correlated with post-bronchodilator FEV_1 % predicted (r=-0.3; p=0.026) and post-bronchodilator FEV_1/FVC (r=-0.35; p=0.008). There was no significant correlation between airway inflammation or the other airway remodelling markers in bronchial biopsies and spirometry measurements (Table 3.3). Smoking history including pack-years was not associated with any of the remodelling or inflammatory features in bronchial biopsies.

3.4.2. CT-derived quantitative morphometry and densitometry univariate correlation with lung function

Subjects with versus those without persistent airflow limitation had significantly narrower mean segmental bronchial luminal areas (9.7 (2.2) versus 11.0 (2.3) mm^2/m^2; p=0.047) and larger mean segmental bronchial wall area % (63.6 (2.0)

versus 62.5 (2.1) %; p=0.039) (Table 3.2). These differences were more marked in the lower versus upper lobe bronchi (Table 3.4). There was significantly more air-trapping in those with versus without persistent airflow limitation as measured by MLD E/I (0.89 [0.05] versus 0.83 [0.05]; p<0.001), and VI-856 HU (%) (32.2 [19.8] versus 15.5 [10.1]; p<0.001). (See Table 3.2).

3.4.3. Univariate correlations between bronchial biopsy airway remodelling and QCT morphometry and air-trapping

Epithelial thickness was significantly correlated with mean segmental bronchial luminal area (r=-0.35; p=0.02), mean segmental bronchial wall area (r=-0.31; p=0.039) and mean segmental bronchial wall area % (r=0.35; p=0.018) (Figure 3.3). Similarly, airway smooth muscle % correlated significantly with mean segmental bronchial luminal area (r=-0.35; p=0.008), mean segmental bronchial wall area (r=-0.32; p=0.015) and mean segmental bronchial wall area % (r=0.27; p=0.045). All the other remodelling and inflammatory markers including vascularity, RBM and inflammatory cell counts in the lamina propria did not have any significant correlation with morphometry indices (Table 3.3).

Vascularity was strongly correlated with measures of air trapping MLD E/I (r=0.49; p<0.001) and VI-856 HU (r=0.53; p<0.001). Airway smooth muscle % was also correlated with MLD E/I (r=0.3; p=0.03) and VI-856 HU (r=0.55; p<0.001) (Figure 3.4).

Epithelial integrity was not correlated any QCT metric. Goblet cell count was only possible to measure in 19 subjects only. There was significant correlation between goblet cell counts per area of intact epithelium and both MLD E/I (r=0.74; p<0.001) and VI-856 HU (r=0.57; p<0.013) (Figure 3.5).

3.4.4. Multivariate analysis of the association between bronchial biopsy immunohistology, lung function and QCT parameters

All airway remodelling and inflammation variables were included in a step-wise multiple regression analysis to examine the predictors of persistent airflow limitation, QCT segmental morphometry and air-trapping. Only airway smooth muscle % was an independent predictor of post-bronchodilator FEV_1 % predicted (R^2=0.24, *β*=-0.49, p=0.001), while both airway smooth muscle % and vascularity were significant predictors of post-bronchodilator FEV_1/FVC (R^2=0.19, *β*=-0.40, p=0.003 and R^2=0.09, *β*=-0.31, p=0.026) respectively). Epithelial thickness and airway smooth muscle % were predictors of mean segmental bronchial luminal area (R^2=0.12, *β*=-035, p=0.02 and R^2=0.12, *β*=-0.35, p=0.015), and wall area (R^2=0.10, *β*=-0.32, p=0.033 and R^2= 0.10, *β*=0.31, p=0.032). Epithelial thickness was the only independent predictor of mean segmental bronchial wall area % (R^2=0.13, *β*=0.35, p=0.018). Vascularity was the only predictor of MLD E/I (R^2=0.24, *β*=0.49, p=0.001), while airway smooth muscle %, vascularity and epithelial thickness all significantly contributed to a model predicting VI-856 HU (R^2=0.31, *β*=0.49, p<0.001; R^2=0.22, β=0.54, p<0.001 and R^2=0.05; *β*=0.24, p=0.045 respectively).

3.4.5. Validation group: replication of the correlation between vascularity and air-trapping

Vascularity in the bronchial biopsies was the only independent predictor of MLD E/I. Therefore the relationship between vascularity and MLD E/I was measured in an independent group of asthmatics (n=24). Baseline demographics and clinical characteristics of subjects in the validation group are described in Table 3.6. Similar to the primary study group, vascularity was positively correlated with MLD E/I (r=0.44; p=0.031) as well as VI-856 HU (r=0.50; p=0.014) (Figure 3.6).

3.5. Discussion

We report here the associations in asthma between bronchial biopsy-derived features of airway inflammation and remodelling with lung function and QCT parameters of proximal airway morphometry and air trapping. We found that neither airway inflammation nor RBM thickening were related to lung function and QCT parameters. However, airway smooth muscle % and vascularity were both associated with airflow obstruction. Proximal airway morphometry was most strongly associated with epithelial thickness and airway smooth muscle % and air-trapping was related to vascularity. This is the first study to suggest a relationship between airway vascularity and air-trapping. However, we are confident that this observation is robust as we were able to confirm this finding in an independent replication group.

Previous studies have explored the relationship between bronchial biopsy features of remodelling and both FEV_1 % predicted and FEV_1/FVC (reviewed in [22]). As reported here airway smooth muscle mass is typically [22,41], but not always [317], a major determinant of lung function impairment. Increased airway smooth muscle mass is a feature of severe childhood asthma [310] and is described in both the large and small airways in studies of asthma deaths [311,312]. Indeed increased airway smooth muscle mass in both the large and small airways is more common than in the large or small airway alone [312]. The Chalkley count method used to assess vascularity in this study is a stereological method used commonly in cancer studies and is well validated for inter- and intra-observer variability, disease progression and mortality [318,319]. Furthermore, the method has been previously used to measure vascular remodelling in asthma with studies showing greater mean Chalkley counts in asthmatics compared to control and correlation with asthma severity [81]. Increased airway vascularity, neoangiogensis, has been consistently reported in endobronchial biopsies from asthmatics compared to healthy controls and in the small airways from lung resections for lung nodules in subjects with asthma [80,81,150,173]. However, increased vascularity was not a feature observed in fatal asthma [320]. We and

others have reported that increased vascularity is associated with lung function impairment [81] and confirmed this finding in the current study. The relationship between airway inflammation and lung function impairment is more contentious with some reports suggesting an association whereas others have not been able to reveal associations (reviewed in [22]). Interestingly in our study, other features of remodelling namely epithelial thickening, RBM thickening and airway inflammation were not associated with lung function indices [22].

Proximal airway morphometry assessed by QCT is abnormal in asthma with luminal narrowing and airway wall thickening [314]. These changes are weakly associated with lung function impairment. We found that epithelial thickening and airway smooth muscle % were related to QCT airway morphometry features of remodelling as described previously [74,127,134], but not other bronchial biopsy measures of remodelling or inflammation. Interestingly, although airway vascularity was associated with lung function impairment it was not associated with proximal airway morphometry.

We have extended previous studies of the relationship between endobronchial features of remodelling and QCT parameters to include measures of air trapping. We found that both airway smooth muscle % and vascularity were associated with air-trapping in univariate analysis, but that vascularity alone was an independent and significant predictor of MLD E/I in our step wise linear regression. In comparative studies of asthma and chronic obstructive pulmonary disease we found that QCT measures of air-trapping are stronger predictors of lung function impairment than changes in proximal airway morphometry [315]. It is therefore intriguing that increased vascularity measured in the proximal airway is related to air trapping a measure of small airway dysfunction. Previous studies suggest that the degree of vascularity in the proximal airway tracks with findings in the small airway [80,320], but of note we did not directly measure remodelling from small airway samples. Due to the novelty of our finding we sought to validate our finding in an independent

replication group. In spite of differences in the processing of the endobronchial biopsies we found a remarkably similar relationship between airway vascularity and QCT-derived air-trapping in the replication group compared with our initial analyses.

Taken together these data support an important role for airway smooth muscle mass in proximal airway remodelling and possibly to a lesser extent in the smaller airway with both likely to be contributing to lung function impairment. Epithelial thickness plays a role in proximal airway remodelling, but is not related to airway dysfunction. Airway vascularity is not associated with proximal airway remodelling, but is associated with air trapping and lung function impairment. Whether increased vascularity promotes small airway closure secondary to oedema or due to direct effects upon airway wall thickness is unknown. Interestingly, there are no reports of effects of corticosteroids upon airway smooth muscle mass, whereas in most although not all studies of the effects of corticosteroids upon airway vasculature demonstrate a decrease in vascularity with a concomitant improvement in lung function [150,172,173,175]. Our study subjects were all receiving inhaled corticosteroid therapy suggesting that the remaining vascularity is resistant to corticosteroid therapy. Whether improvements in airway vascularity in response to corticosteroid or other therapies are related to improvements in air-trapping requires further study.

The relationship between goblet cell counts and air trapping is intriguing. Although there was no correlation of any epithelial measured remodelling change other than epithelial thickness with any morphometry markers, goblet cell hyperplasia very strongly correlated with both MLD E/I and VI-856 HU. However, the number of subjects with sufficient intact epithelium to allow enumeration of goblet cells was only 19, thus these results need to be interpreted with caution. For the same reason, goblet cell counts were not included in the multivariate regression analysis.

This study has a number of potential limitations. Although this is the largest study to date comparing immunohistology with QCT parameters of airway remodelling it

remains a relatively small study. It is also cross-sectional and future longitudinal studies of the natural history of asthma and response to therapies should consider inclusion of endobronchial biopsy and imaging parameters to further determine the structure-function relationships. Importantly, we did not standardise the location of the sampling of the endobronchial biopsies with a corresponding airway identified by QCT and whether this is important to determine the heterogeneity within an individual will be important in future studies. However, we did reduce the variability of QCT parameters within an individual by using the mean airway morphometry derived from multiple airways and demonstrated good within donor repeatability of the key structural wall components in bronchial biopsies. Critically, our comparisons between QCT air-trapping were with proximal rather than distal airway samples. As discussed above it is likely that these proximal airway samples reflected similar changes in the smaller airways, but notwithstanding this likelihood further studies are required to compare QCT parameters of the small airway with distal sampling such as transbronchial biopsies. In contrast to Kasahara and colleagues our study has shown no correlation between RBM and QCT-derived morphometry parameters [74,129]. However, similar to our observation, Saglani *et al* also failed to demonstrate such correlation [129]. Another limitation of this study relates to using endobronchial biopsies to measure airway remodelling. These biopsies are small and only sample the superficial layer of the airway, thus cannot determine the changes in airway structure in relationship to the whole depth of the airway wall. However, despite this shortcoming, endobronchial biopsies remain the best in vivo tool to assess the structural changes in the airway wall contributing to airway remodelling [303].

In conclusion, we have found important associations between endobronchial biopsy and QCT measures of airway remodelling with lung function. We found that airway smooth muscle mass and airway vascularity are related to airflow obstruction with airway smooth muscle mass likely contributing more to large than small airway

remodelling, whereas increased vascularity appears to be related to air trapping possibly due to small airway remodelling.

Table 3.1: Demographics, clinical and laboratory characteristics

Characteristic		**All patients (n = 70)**	**Post-bronchodilator FEV_1<80% (n =30)**	**Post-bronchodilator FEV_1≥80% (n = 40)**	***p*-value**
Age (y)		49 (12)	52 (12)	47 (13)	0.095
Male (%)		57	67	50	0.163
Caucasian (%)		93	93	93	0.893
Asthma duration (y)		24 (18)	29 (20)	20 (15)	0.059
BMI (kg/m^2)		29.9 (5.6)	30.3 (5.9)	29.6 (5.4)	0.644
Ex-smokers (%)		19	27	13	0.131
Smoking pack-years		0.0 [0.0-0.0]	0.0 [0.0-3.9]	0.0 [0.0-0.0]	0.109
Atopy (%)		81	77	81	0.659
GINA class	**GINA 5, n (%)**	22 (31)	13 (43.3)	9 (23)	0.084
	GINA 4, n (%)	34 (49)	15 (50.0)	19 (48)	
	GINA 3, n (%)	6 (9)	0 (0.0)	6 (15)	
	GINA 1&2, n (%)	8 (11)	2 (6.7)	6 (16)	
Inhaled BDP equivalent (µg/24h)		1289 (689)	1444 (658)	1173 (698)	0.104
Pre-Bronchodilator FEV_1 (L)		2.46 (0.92)	1.76 (0.65)	2.98 (0.73)	**<0.001**
Pre-Bronchodilator FEV_1 (% predicted)		78.8 (24.6)	55.1 (14.3)	96.5 (12.8)	**<0.001**
Pre-Bronchodilator FEV_1/FVC (%)		66.7 (13.3)	55.7 (11.2)	74.9 (7.7)	**<0.001**
Post-Bronchodilator FEV_1 (L)		2.63 (0.91)	1.98 (0.63)	3.14 (0.77)	**<0.001**
Post-Bronchodilator FEV_1 (% predicted)		84.8 (23.3)	62.1 (12.8)	101.8 (12.0)	**<0.001**
Post-Bronchodilator FEV_1/FVC (%)		69.5 (12.5)	59.3 (11.0)	77.1 (6.8)	**<0.001**

Characteristic	All patients (n = 70)	Post-bronchodilator FEV_1<80% (n =30)	Post-bronchodilator FEV_1≥80% (n = 40)	*p*-value
Induced sputum				
Sputum eosinophils (%)[#]	4.5 [1.4-18.8]	5.3 [2.0-23.1]	4.2 [0.03-10.0]	0.185
Sputum neutrophils (%)[#]	46.5 [25.6-63.5]	49.7 [36.8 -68.4]	44.1 [17.6-63.2]	0.066
Immunohistochemistry				
Tissue eosinophils (cells/mm^2 of lamina propria) [#]	19.6 [8.0-32.7]	19.2 [8.3-35.4]	19.9 [8.0-28.3]	0.947
Tissue neutrophils (cells/mm^2 of lamina propria) [#]	5.8 [2.2-20.8]	4.3 [2.1-15.5]	9.6 [2.3-24.6]	0.924
Tissue mast cells (cells/mm^2 of lamina propria) [#]	15.7 [5.4-33.6]	13.8 [6.2-37.1]	15.7 [5.3-22.9]	0.149
RBM thickness (µm)	12.3 (3.9)	12.3 (4.4)	12.3 (3.6)	0.974
Airway smooth muscle %	25.8 (15.4)	33.5 (15.6)	20.1 (12.6)	**<0.001**
Vascularity (mean Chalkley count)	5.5 (1.8)	6.2 (1.6)	5.0 (1.9)	**0.017**
Epithelial Thickness (µm)	62.0 (16.8)	65.1 (17.5)	59.7 (16.2)	0.257
Intact epithelium % [#]	27.8 [12.5-49.7]	36.2 [15.2-54.2]	22.9 [9.8-45.0]	0.466

Mean (SD) unless stated; [#] *median [IQR]. BDP- Beclomethasone dipropionate*

Table 3.2: QCT morphometry and air-trapping parameters

Characteristic	**All patients (n = 56)**	**Post-bronchodilator FEV_1<80% (n =29)**	**Post-bronchodilator FEV_1≥80% (n = 25)**	***p*-value**
CT-derived quantitative morphometry				
Mean segmental bronchial lumen area/BSA (mm^2/m^2)	10.4 (2.3)	9.7 (2.2)	11.0 (2.3)	**0.047**
Mean segmental bronchial wall area/BSA (mm^2/m^2)	17.0 (2.6)	16.5 (2.9)	17.5 (2.4)	0.133
Mean segmental bronchi wall area %	63.0 (2.2)	63.6 (2.0)	62.5 (2.1)	**0.039**
CT-derived measures of air-trapping				
MLD E/I	0.85 (0.06)	0.89 (0.05)	0.83 (0.05)	**<0.001**
VI-856HU (%)	22.6 (17.0)	32.2 (19.8)	15.5 (10.1)	**<0.001**

*Data are presented as mean±SD, unless otherwise stated. FEV1: forced expiratory volume in 1 s; BSA: body surface area; MLD E/I: mean lung density expiratory/inspiratory ratio on the expiratory/inspiratory scan; VI-856HU: percentage of lung voxels with a density lower than –856 HU on expiratory scans. *: p<0.05.*

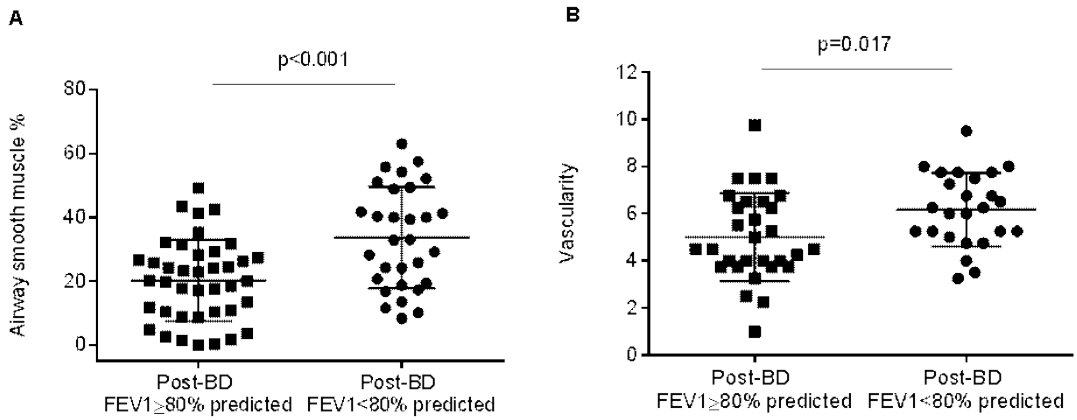

Figure 3.1: A) Airway smooth muscle % and B) vascularity (mean Chalkley count) in subjects with and without persistent airflow limitation (post-bronchodilator [BD] FEV_1 <80% and ≥80% predicted).

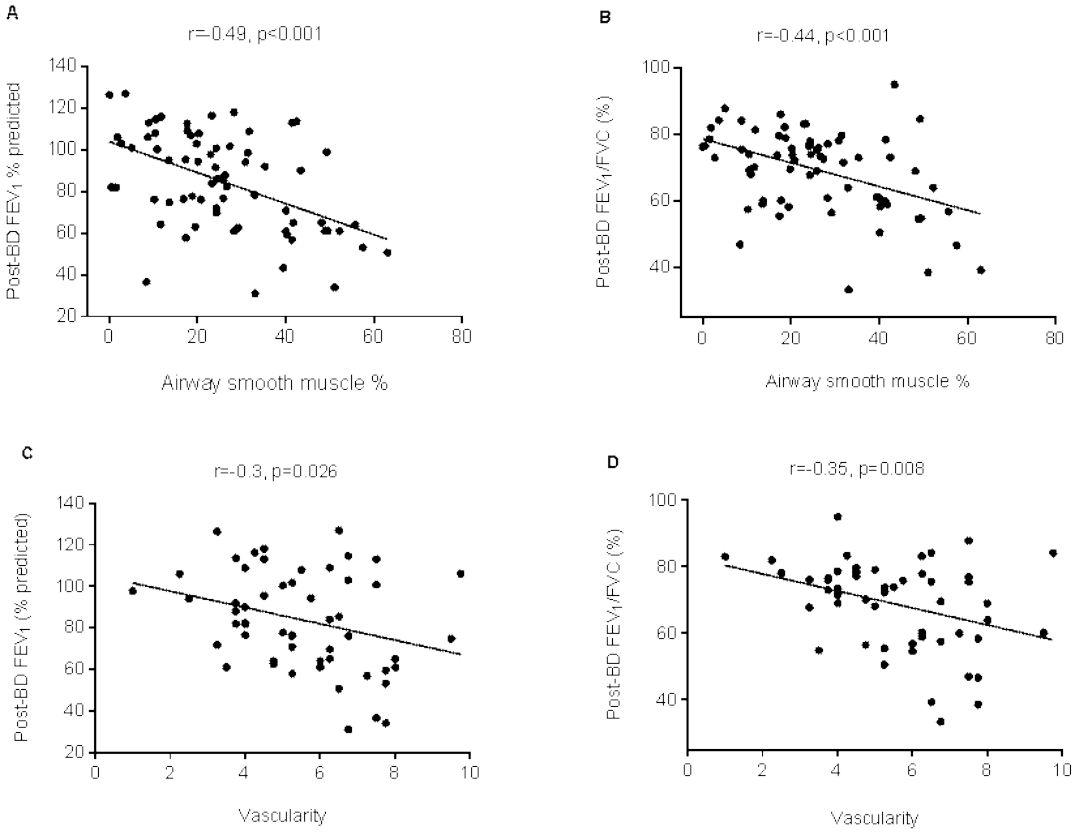

Figure 3.2: Scatterplots showing correlations of post-bronchodilator [BD] FEV_1 and FEV_1/FVC with a) and b) airway smooth muscle % and c) and d) vascularity.

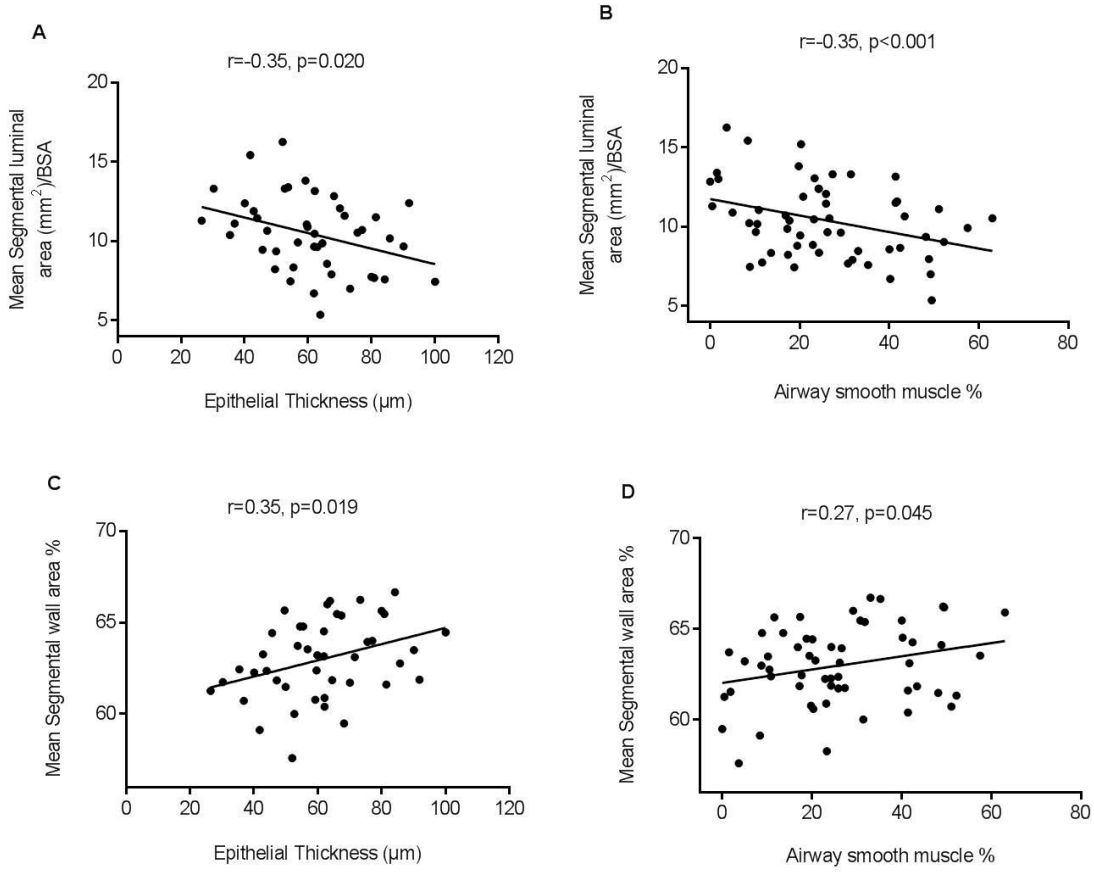

Figure 3.3: Scatterplots showing correlations of epithelial thickness and airway smooth muscle % with a) and b) mean segmental bronchial luminal and c) and d) mean segmental wall area %.

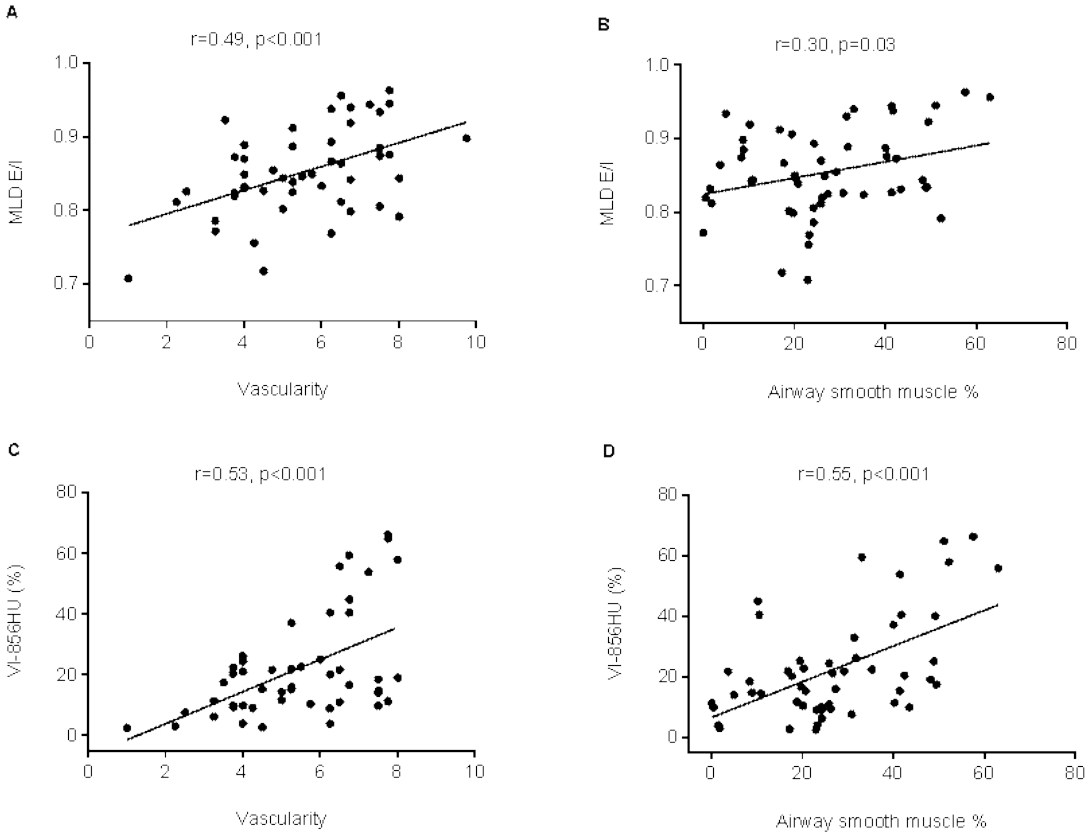

Figure 3.4: Scatterplots showing correlations of vascularity and airway smooth muscle % with a) and b) MLD E/I and c) and d) VI-856 HU.

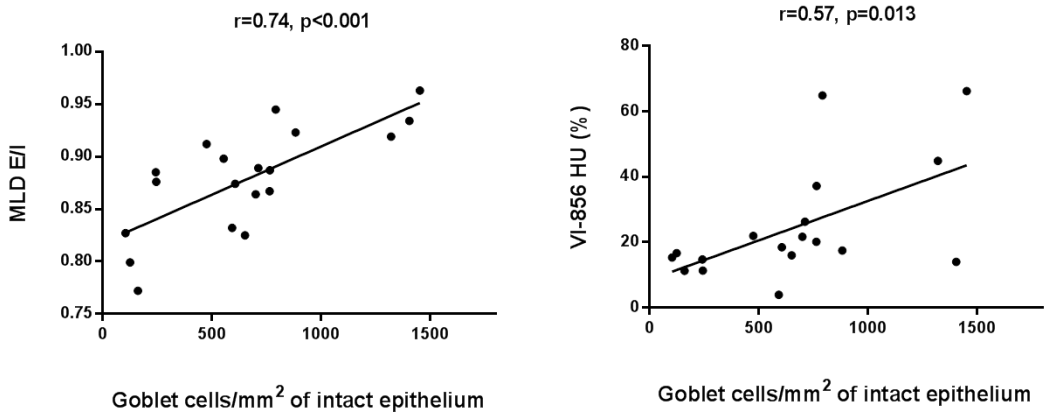

Figure 3.5: Scatter plot showing correlation of Goblet cells count and QCT air-trapping markers.

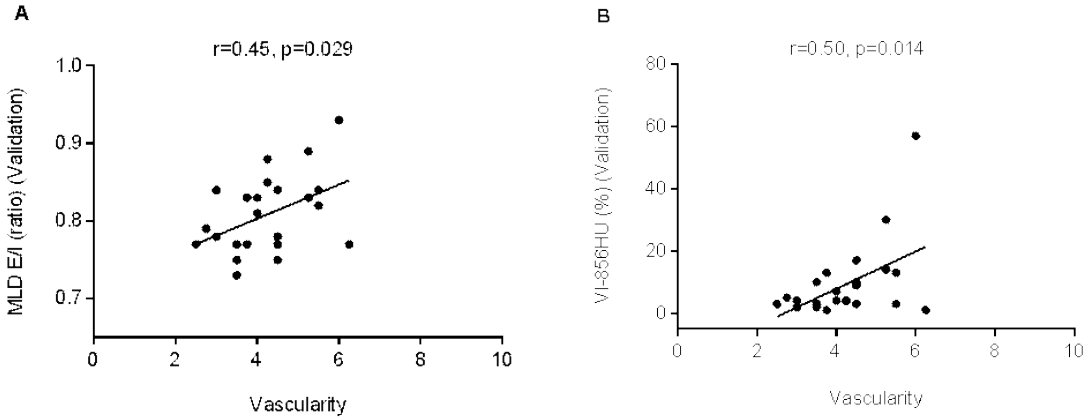

Figure 3.6: Validation of the association between vascularity and air-trapping in the replication group showing scatterplots of vascularity with a) MLD E/I and b) VI-856 HU.

Table 3.3: Univariate correlations between primary QCT parameters and lung function, airway inflammation and remodelling

	Submucosal eosinophils #	Submucosal neutrophils #	Submucosal mast cells #	Airway smooth muscle %	RBM thickness	Submucosal Vascularity	Epithelial Thickness	Intact epithelium%
Mean segmental LA/BSA (mm2/m2)	-0.26	0.05	-0.13	-0.35**	-0.13	0.06	-0.35*	0.04
Mean segmental WA/BSA (mm2/m2)	-0.27	-0.02	-0.14	-0.32*	-0.18	0.04	-0.31*	0.06
Mean segmental %WA	0.22	-0.28	0.10	0.27*	-0.01	-0.09	0.35*	-0.08
MLD E/I	0.03	-0.31	-0.15	0.3*	0.05	0.49***	0.07	-0.09
VI-856HU	-0.06	-0.17	0.00	0.55***	0.07	0.53***	0.07	-0.09
Post-BD FEV1 (%pred)	-.03	0.13	-0.18	-0.49***	-0.01	-0.30*	0.00	0.06
Post-BD FEV/FVC (%)	0.05	0.25	0.02	-0.44***	0.00	-0.35**	0.08	0.00

Pearson's correlation coefficient for parametric data, otherwise # Spearman's correlation coefficient for non-parametric data.

p value <0.05[*], <0.01[**] p value<0.001[***]

Table 3.4: Segmental bronchi Morphometry markers in subjects with and without fixed airflow obstruction

Characteristic	All patient	Post-bronchodilator FEV_1<80%	Post-bronchodilator FEV_1≥80%	*p*-value
RB1 lumen area/BSA (mm^2/m^2)	10.2 (3.7)	10.3 (3.6)	10.3 (3.5)	0.858
RB1 wall area percentage	64.0 (4.3)	63.9 (4.2)	64.0 (4.2)	0.718
RB2 LA/BSA (mm^2/m^2)	10.4 (3.0)	10.2 (3.0)	10.6 (3.1)	0.667
RB2 wall area percentage	62.5 (3.3)	62.9 (3.3)	62.1 (3.3)	0.406
RB3 LA/BSA (mm^2/m^2)	14.7 (6.6)	14.0 (5.4)	15.3 (7.5)	0.496
RB3 wall area percentage	60.3 (3.7)	60.5 (4.5)	60.2 (3.0)	0.817
RB4 LA/BSA (mm^2/m^2)	8.4 (3.7)	7.7 (3.3)	9.0 (3.9)	0.211
RB4 wall area percentage	63.1 (4.2)	63.6 (4.3)	62.8 (4.1)	0.474
RB5 LA/BSA (mm^2/m^2)	9.3 (3.3)	8.6 (2.8)	9.8 (3.6)	0.184
RB5 wall area percentage	62.9 (3.1)	63.6 (2.7)	62.3 (3.3)	0.12
RB6 LA/BSA (mm^2/m^2)	12.8 (5.0)	11.1 (4.1)	14.2 (5.3)	**0.024**
RB6 wall area percentage	62.3 (4.3)	63.0 (4.0)	61.3 (4.5)	0.261
RB7 LA/BSA (mm^2/m^2)	8.2 (3.1)	7.6 (2.8)	8.7 (3.4)	0.203
RB7 wall area percentage	65.1 (3.4)	65.8 (3.5)	64.4 (3.2)	0.165
RB8 LA/BSA (mm^2/m^2)	9.7 (2.9)	8.6 (2.4)	10.5 (3.0)	**0.013**

Characteristic	All patient	Post-bronchodilator FEV_1<80%	Post-bronchodilator FEV_1≥80%	*p*-value
RB8 wall area percentage	63.3 (3.2)	64.5 (2.7)	62.4 (3.4)	0.023
RB9 LA/BSA (mm^2/m^2)	9.0 (4.1)	8.4 (3.7)	9.5 (4.4)	0.303
RB9 wall area percentage	63.9 (3.8)	64.3 (3.1)	63.5 (4.1)	0.422
RB10 LA/BSA (mm^2/m^2)	11.4 (3.8)	10.1 (3.2)	12.5 (3.9)	**0.015**
RB10 wall area percentage	61.9 (3.2)	63.2 (3.1)	60.8 (3.1)	**0.006**
LB1 LA/BSA (mm^2/m^2)	8.6 (2.7)	9.4 (2.9)	8.1 (2.4)	0.097
LB1 wall area percentage	64.1 (2.9)	63.5 (3.2)	64.6 (2.8)	0.199
LB2 LA/BSA (mm^2/m^2)	6.3 (3.2)	6.5 (3.2)	6.2 (3.2)	0.796
LB2 wall area percentage	64.7 (3.8)	64.6 (3.6)	64.7 (4.0)	0.96
LB3 LA/BSA (mm^2/m^2)	12.7 (4.9)	13.1 (5.4)	12.4 (4.6)	0.627
LB3 wall area percentage	61.3 (4.4)	61.9 (5.0)	60.9 (3.9)	0.414
LB4 LA/BSA (mm^2/m^2)	7.9 (3.2)	8.2 (3.6)	7.6 (2.9)	0.498
LB4 wall area percentage	62.7 (4.2)	62.6 (4.5)	62.8 (4.1)	0.874
LB5 LA/BSA (mm^2/m^2)	7.1 (2.0)	6.6 (1.8)	7.4 (2.2)	0.161
LB5 wall area percentage	64.2 (2.7)	65.2 (2.5)	63.4 (2.5)	**0.015**
LB6 LA/BSA (mm^2/m^2)	15.2 (6.4)	12.8 (5.1)	17.2 (6.8)	**0.009**
LB6 wall area percentage	61.0 (4.7)	62.2 (4.4)	59.9 (4.7)	0.068

Characteristic	All patient	Post-bronchodilator FEV_1<80%	Post-bronchodilator FEV_1≥80%	*p*-value
LB1+2 LA/BSA (mm^2/m^2)	13.4 (5.0)	12.4 (4.9)	14.1 (5.0)	0.216
LB1+2 wall area percentage	62.3 (4.2)	63.1 (4.8)	61.6 (3.7)	0.219
LB8 LA/BSA (mm^2/m^2)	11.5 (4.1)	11.1 (5.0)	11.9 n(3.4)	0.511
LB8 wall area percentage	63.5 (3.5)	64.7 (3.5)	62.7 (3.4)	**0.042**
LB9 LA/BSA (mm^2/m^2)	9.4 (3.7)	8.4 (3.5)	10.1 (3.7)	0.107
LB9 wall area percentage	64.5 (3.1)	65.3 (3.1)	63.9 (3.1)	0.105
LB10 LA/BSA (mm^2/m^2)	11.2 (3.6)	9.7 (2.6)	12.3 (3.9)	**0.009**
LB10 wall area percentage	62.0 (3.1)	62.8 (3.0)	61.4 (3.2)	0.103

Table 3.5: Demographics, clinical and laboratory characteristics of the Chalkley validation group.

Characteristic		**Subjects (n = 24)**
Age (y)		33.8 (11.6)
Male (%)		37.5
Caucasian (%)		42.1
Asthma duration (y)		20.1 (11.1)
BMI (kg/m^2)		28.9 (5.7)
Age of asthma onset (y)		15.5 (14.3)
Ex-smokers (%)		4.2
Atopy (%)		79.2
GINA class	**GINA 5 (%)**	16.7
	GINA 4 (%)	50
	GINA 3 (%)	0
	GINA 1&2 (%)	33.3
Inhaled BDP equivalent (µg/24h)		1506 (938)
Pre-Bronchodilator FEV_1 (L)		2.7 (0.9)
Pre-Bronchodilator FEV_1 (% predicted)		83.1 (17.5)
Pre-Bronchodilator FEV_1/FVC (%)		83.8 (10.4)
Post-Bronchodilator FEV_1 (L)		3.0 (0.9)
Post-Bronchodilator FEV_1 (% predicted)		92.0 (13.7)
Post-Bronchodilator FEV_1/FVC (%)		88.1 (10.3)
Vascularity (mean Chalkley count)		4.2 (1.0)
MLD E/I		0.81 (0.05)
€VI-856HU		9.1 (12.1)

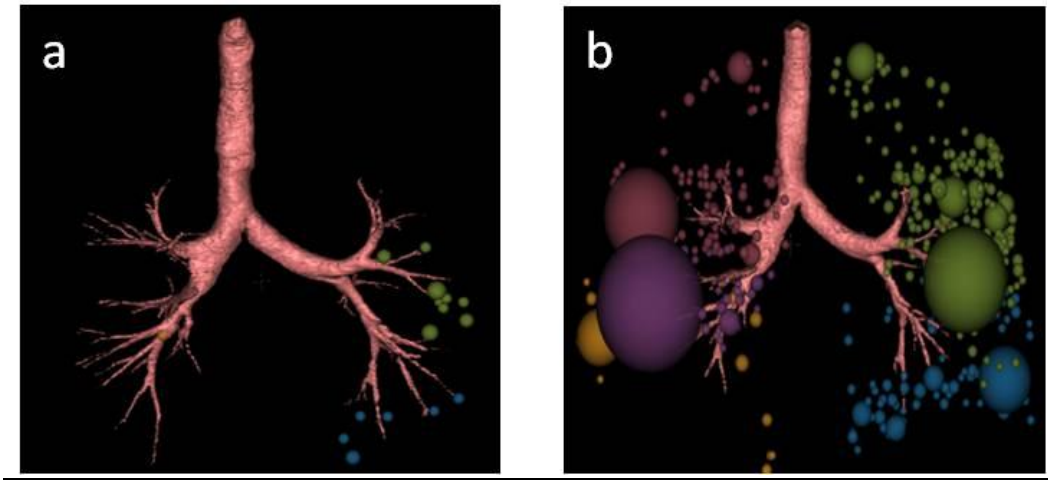

Figure 3.7: A representative inspiratory and expiratory CT scan from a subject with asthma illustrating the low attenuating clusters a) <950 HU in inspiration, and b) <856 in expiration.

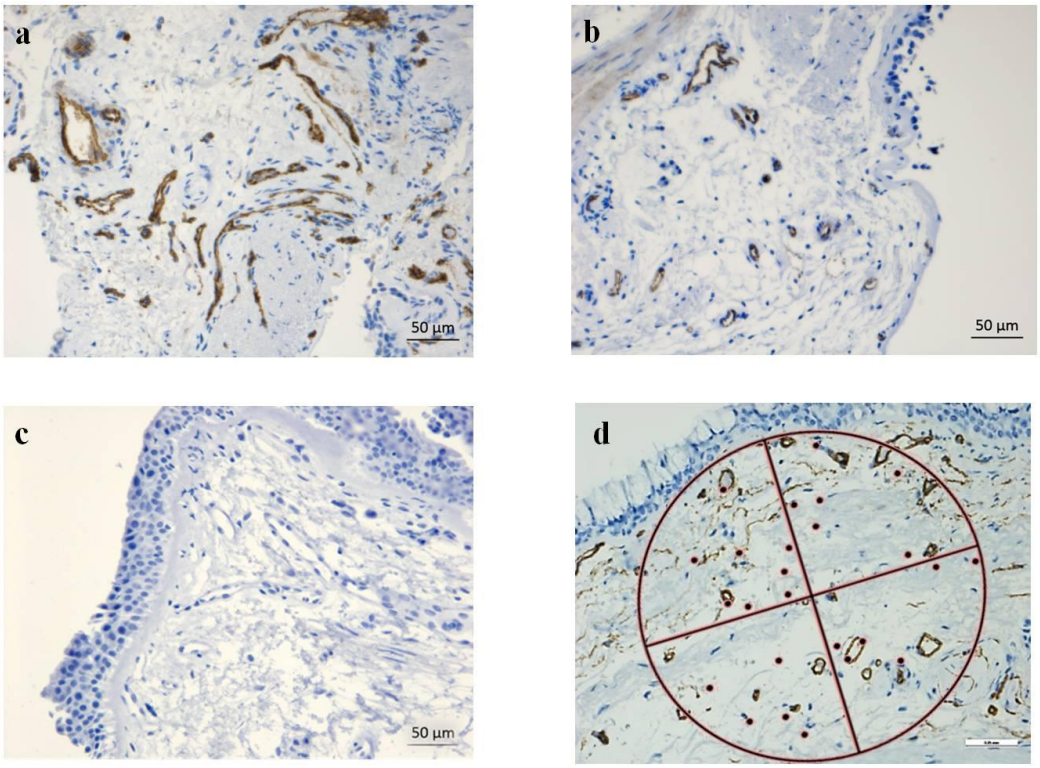

Figure3.8: Examples of endobronchial biopsy sections; a) anti-endothelium (EN4) staining in a section with high Chalkley count , b) EN4 staining in a section with low Chalkley count, c) negative isotype control, and d) an example of a vascular hot spot on a EN4 stained section with superimposed point array from a Chalkley graticule normally fixed to the microscope eye piece. In each hotspot, after both rotating the graticule and moving the section, the Chalkley count is obtained by counting the maximum number of dots touching stained vessels.

4. CHAPTER FOUR: THE EFFECTS OF PROSTAGLANDIN D2 RECEPTOR 2 ANTAGONISM ON AIRWAY INFLAMMATION, ASTHMA SYMPTOMS, LUNG FUNCTION AND AIRWAY REMODELLING IN UNCONTROLLED MODERATE-SEVERE EOSINOPHILIC ASTHMA

4.1. Abstract

Background: Eosinophilic airway inflammation is often present in asthma and interventions that reduce it result in improved clinical outcomes. Antagonism of the prostaglandin D_2 receptor 2 (DP_2) may reduce eosinophilic airway inflammation and alter airway remodelling.

Methods: We performed a single-centre, 12-week, randomized, double-blind, placebo-controlled, parallel-group clinical trial of the DP_2 receptor antagonist fevipiprant (FEVIPIPRANT) 225mg twice per day orally in 61 subjects with persistent moderate-to-severe asthma and an elevated sputum eosinophil count. The primary outcome was the change in sputum eosinophil percentage from baseline to post-treatment. Secondary and exploratory outcomes included changes in Asthma Control Questionnaire score (ACQ-7), standardised Asthma Quality of Life Score (AQLQ(S)), forced expiratory volume in one second (FEV_1), bronchial submucosal inflammation, and airway remodelling as measured on bronchial biopsies. This trial is registered with ClinicalTrials.gov (NCT01545726).

Results: Sputum eosinophil percentage fell from a geometric mean of 5·4% at baseline to 1·1% post-treatment in the fevipiprant group and from 4·7% at baseline to 3·9% post-treatment in the placebo group (between group difference 3·5-fold; 95% confidence interval 1·7 to 7·0; p = 0·0014). Bronchial submucosal eosinophils were reduced 2.5-fold in the fevipiprant group compared to placebo (p = 0.040). ACQ-7 score fell by 0·32 points in the fevipiprant group compared to placebo (p = 0·17) and

by 0.56 points in the subgroup with poor control (≥1.5 points) at baseline (p = 0.046). In the fevipiprant group compared to placebo AQLQ(S) improved by 0.59 points (p = 0.0080) and post-bronchodilator FEV_1 improved by 0.16 L (p = 0.021). Patients treated with fevipiprant had significant improvement in epithelial integrity and reduction in airway smooth muscle mass compared to placebo.

Fevipiprant displayed a favourable safety profile, with no serious adverse events reported.

Conclusion: Fevipiprant reduces eosinophilic airway inflammation in patients with persistent asthma and raised sputum eosinophil counts despite inhaled corticosteroid treatment. The treatment also improves epithelial and airway smooth muscle remodelling. All this is associated with improved lung function and asthma-related quality of life, and a favourable safety profile.

4.2. Introduction

Asthma is a chronic inflammatory airway disease that is characterised by heterogeneity with respect to clinical phenotype and response to therapy [321]. Eosinophilic airway inflammation, mediated by type 2 immunity, is a common feature of asthma [321]. Treatment strategies that specifically target eosinophilic airway inflammation substantially reduce exacerbations of asthma in those patients with uncontrolled eosinophilic airway inflammation, and to a lesser extent improve lung function and asthma control [227,229,239,243,322,323].

There is increasing evidence that prostaglandin D2 (PGD_2), acting upon the DP_2 receptor, also known as receptor homologous molecule expressed on T-helper 2 cells ($CRTH_2$), may play an important role in mediating eosinophilic airway inflammation in asthma. The DP_2 receptor mediates the migration of T-helper 2 (Th_2) cells, delays their apoptosis and stimulates them to produce the cytokines IL-4, IL-5 and IL-13 [25,324,325]. DP_2 also influences the migration of and cytokine release from type 2 innate lymphoid cells [326], and importantly the receptor is expressed by eosinophils, and directly mediates their chemotaxis and degranulation [327,328]. The number of DP2+ cells in the bronchial submucosa increases with increasing severity of asthma [277]. DP_2 is also expressed on airway epithelial cells and directly promotes their migration and differentiation [277]. DP_2 is therefore a highly promising novel drug target in the treatment of asthma. Fevipiprant (FEVIPIPRANT) is an orally administered highly selective and potent antagonist of the DP_2 receptor, but not to the more general homeostatic PGD_2 receptor DP_1.

We tested the hypothesis that, in patients with sputum eosinophilia (≥ 2%) and persistent, moderate-to-severe asthma, 12-weeks' treatment with fevipiprant at a dose of 225mg twice per day, on top of conventional treatment, reduces the levels of eosinophils in induced sputum compared to placebo. Secondary objectives were to determine the effects of fevipiprant on asthma symptoms, as measured by the seven-point Asthma Control Questionnaire (ACQ-7) [300,301], and to assess safety

and tolerability of fevipiprant. Exploratory objectives included assessment of the effect of fevipiprant on the forced expiratory volume in one second (FEV_1), lung volumes using body plethysmography, health-related quality of life as measured by the standardised Asthma Quality of Life Questionnaire (AQLQ(S)) [300], airway inflammation and remodelling in bronchial biopsies and airway morphometry and lung density assessed by quantitative computed tomography (QCT).

4.3. Methods

4.3.1. Subjects

Participants were older than 18 years of age and had a clinical diagnosis of asthma that was supported by one or more of the following criteria: an increase in forced expiratory volume (FEV_1) of ≥ 12% and ≥ 200ml from its pre-bronchodilator value following the inhalation of 400µg salbutamol, a provoked fall in FEV_1 of 20% by methacholine at ≤ 16mg/ml while on inhaled corticosteroids (ICS), or a change in FEV_1 of > 12% over two non-exacerbation-related measurements during the previous year.

Participants were recruited from a regional refractory asthma clinic providing tertiary care for a population of 4 million people. Suitable participants were also identified from secondary care asthma and general respiratory clinics in the region, and through screening of local primary care databases. Inclusion criteria were current treatment with ICS, a sputum eosinophil count of ≥ 2% at screening, and either an Asthma Control Questionnaire (ACQ-7) score ≥ 1·5 at randomization or ≥ 1 exacerbations (requiring higher than the patient's normal dose of systemic corticosteroids for ≥ 3 days) in the past 12 months. Exclusion criteria included serious coexisting illness, pregnancy or lactation, the possibility of conception, a history of malignancy within the previous five years, recent (within 6 weeks of screening) lower respiratory tract infection or exacerbation of asthma requiring oral prednisolone, the use of omalizumab within 6 months before randomization into the study, and the use of immunosuppressive medication (except low-dose [≤ 10mg prednisolone per day]

oral corticosteroids) within 30 days before randomization. All subjects provided written informed consent. The study protocol was approved by the National Research Ethics Committee (Leicestershire, Northamptonshire and Rutland, approval no. 11/EM/0402) and the United Kingdom Medicines and Healthcare Products Regulatory Agency. The trial was registered with ClinicalTrials.gov (NCT01545726) and EudraCT (2011-004966-13).

4.3.2. Design of the study

The study was a single-centre, randomised, double-blind, placebo-controlled, parallel-group clinical trial conducted from February 2012 through June 2013. The funding organisation (Novartis Pharmaceuticals) supplied the study drug and placebo. The study design is illustrated in Figure 4.1.

Participants were given the option of undergoing bronchoscopy at the baseline and post-treatment visits as part of the study. Patients attended a screening visit (Visit 1, Day -21), at which demographic and clinical details were collected, and inclusion and exclusion criteria were reviewed. An induced sputum sample was collected and cell count was performed, in order to assess eligibility based upon a sputum eosinophil count of ≥ 2%. Regular treatment was kept constant from this time point until the end of the study. One week later, a two-week single-blind placebo run-in period was commenced (Visit 2, Day -14). Following this, patients attended a baseline visit (Visit 3, Day 0), at which they completed the ACQ-7 questionnaire, and eligibility based upon the inclusion and exclusion criteria was again assessed, taking into account the ACQ-7 score. If patients fulfilled the criteria, they proceeded to undertake the remainder of the study visit tests, and were then randomized in a 1:1 ratio to receive either fevipiprant at a dose of 225 mg twice per day, or an identical placebo. Patients attended a mid-treatment visit (Visit 4, Day 42), and a post-treatment visit (Visit 5, Day 84). At the post-treatment visit, patients began a six-week single-blind placebo washout period, and then attended an end-of-study visit (Visit 6, Day 126).

All tests performed at the baseline and post-treatment visits were carried out on the same day, with the exception of bronchoscopy, which was performed on a separate day not more than seven days following the other tests, but not on the day immediately following them, due to the possibility of interaction between the sputum induction procedure and bronchial biopsies. The time interval between the two testing days was kept constant for each patient between the baseline and post-treatment visits. On the first testing day, patients completed the ACQ-7 and Standardized Asthma Quality of Life Questionnaire (AQLQ(S)). The fractional exhaled nitric oxide at 50ml/s ($FeNO_{50}$) was measured using a NIOX MINO device (Aerocrine AB, Solna, Sweden). Patients undertook body plethysmography, measurement of carbon monoxide diffusing capacity and pre-bronchodilator spirometry. An induced sputum sample was then collected and cell count performed. Salbutamol (400 µg via a metered-dose inhaler and spacer) was administered, followed by the measurement of post-bronchodilator spirometry. A blood sample was drawn for the measurement of blood eosinophil count. Inspiratory and expiratory computed tomography (CT) was then performed. On the second testing day, bronchoscopy was performed. Six weeks following randomization, patients attended a mid-treatment visit (Visit 4, Day 42), at which they completed the ACQ-7 and AQLQ(S) questionnaires, pre- and post-bronchodilator spirometry was performed, an induced sputum sample was obtained, and a blood sample was drawn for the measurement of blood eosinophil count. Twelve weeks following randomization, patients attended a post-treatment visit (Visit 5, Day 84), which incorporated the same assessment schedule as the baseline visit. Patients then began a six-week single-blind placebo washout period. Following this, patients attended an end-of-study visit (Visit 6, Day 126) and undertook the same assessments as at the baseline and post-treatment visits, except that bronchoscopy and CT scans were not performed.

Safety was assessed at each study visit on the basis of patient-reported adverse events, physical examination, vital signs, haematology, blood chemistry, urinalysis and an electrocardiogram. Criteria for withdrawal from the study were defined *a*

priori, and included withdrawal of informed consent, asthma exacerbation, pregnancy, and adverse events for which continued exposure to the study drug would be detrimental.

4.3.3. Pulmonary function tests and sputum induction

Spirometry was performed using a rolling seal spirometer (Vitalograph, UK) according to American Thoracic Society / European Respiratory Society (ATS / ERS) guidelines [296], and repeated twenty minutes after inhalation of 400µg salbutamol. Bronchial provocation testing to methacholine was performed using the tidal breathing method as previously described [329]. Methacholine was inhaled to a maximum concentration of 16mg/ml and the PC_{20} calculated by linear interpolation of the log-transformed plot. The procedure was only performed in those in whom no other objective evidence supporting the diagnosis was available. Body plethysmography and carbon monoxide transfer factor were performed according to ATS / ERS guidelines (Medisoft, Belgium) [330,331]. The fractional exhaled nitric oxide was measured using a NIOX MINO device (Aerocrine AB, Solna, Sweden), as previously described [332]. Sputum induction and processing was performed as previously described [298]. Differential cell counts were recorded by a blinded individual and expressed as percentage values of a sample containing at least 400 non-squamous cells.

4.3.4. Skin tests for allergy

Skin prick tests for allergy were performed at Visit 2 to assess atopic status unless historical positive results were available. Patients were tested against a panel of aeroallergens comprising grass pollen, tree pollen [alder, silver birch, hazel], moulds [*Aspergillus fumigatus, Alternaria tenius, Cladosporium, Penicillium notatum*], cat fur, dog dander, and house dust mite [*Dermatophagoides pteronyssimus*. Small droplets of purified allergen extracts were placed on the subject's forearm, as well as a positive control (histamine) and negative control (saline). A sterile lancet was passed

through each droplet so as to break the epidermis of the skin underneath the droplet without drawing blood. A new lancet was used for each droplet, which was then immediately discarded. Surplus fluid was then blotted from the skin. After 15 minutes the diameter of the wheal produced by each allergen was measured. A wheal diameter of 3mm or more than that of the negative control was considered to be a positive result.

4.3.5. Bronchial biopsy analysis

All bronchoscopies were performed by blinded senior clinicians, in accordance with published guidelines [299]. During bronchoscopy, subjects had up to six endobronchial biopsies. Biopsy specimens were processed as previously described and embedded in glycol methacrylate [333].

Two micrometre sections were cut and stained with Haematoxylin & Eosin (H&E) and Periodic acid–Schiff (PAS). Furthermore, immunohistochemical staining was done with the following mAbs: anti–mast cell tryptase clone AA1 (Dako UK, Ely, United Kingdom), anti-human smooth muscle actin (SMA) clone 1A4 (Dako UK, Ely, United Kingdom), anti-eosinophil major basic protein clone BMK-13 (Monosan, Uden, The Netherlands), anti-neutrophil elastase clone NP57 (Dako UK, Ely, United Kingdom), anti-CD3 polyclonal antibody (Becton Dickinson, San Jose, California, USA), anti-endothelium clone EN4 (Monosan, Uden, The Netherlands) and anti-mucin 5AC (MUC5AC) clone 45M1 (Abcam, Cambridge, UK). Appropriate isotype controls were used.

For image analysis and morphometry ZEN 2012 image analysis software for light microscopy (Carl Zeiss AG, Jena, Germany) was used. Tissue section areas were measured in H&E and SMA stained sections. Total area, airway smooth muscle area and epithelial area were measured directly, while lamina propria area was calculated by subtracting the all the other areas and the area occupied by vessels and lymphatics from the total section area. All areas were expressed in mm^2 and also as

percentages of the total area. All morphometry measurements and cellular counts were performed by one blinded observer on two non-contiguous tissue sections at least 20µm apart from the same biopsy block.

Reticular basement membrane (RBM) thickness was measured at x200 magnification by measuring 50 points 20µm apart according to the method validated by Sullivan *et al* [113]. Epithelial thickness was measures using the method described by Cohen *et al* [305]. Briefly, areas of intact and tangentially orientated epithelium were identified and measured. Subsequently, to calculate the epithelial thickness, this area was divided by the lengths of the corresponding RBM. Both RBM and epithelial thickness were expressed in µm. Vascularity was measured using the Chalkley count, a surrogate of both vessel density and vascular area. As described previously, a Chalkley eyepiece graticule (NG52 Chalkley Point Array, Pyser-SGI Ltd, Edenbridge, UK) was used at x200 to measure Chalkley counts in four non-overlapping vascular hotspots (1-2/section) [81]. The mean Chalkley count (MCC) was calculated as the mean of the four measurements. Epithelial integrity was assessed by measuring the lengths of intact epithelial denuded epithelium. These were expressed as percentage of all the RBM length present in the section. For inflammatory cell counts, submucosal nucleated stained inflammatory cells (eosinophils, mast cells and neutrophils) were counted on the corresponding stained sections and expressed at cells/mm^2 of lamina propria.

4.3.6. Computed Tomography

Volumetric whole lung scans were obtained using a Siemens Sensation 16 scanner (16 x 0·75 mm collimation, 1·5 mm pitch, 120 kVp, 40 mAs, 0·5 seconds rotation time and scanning field of view of 500 mm). The scans were obtained at full inspiration (near total lung capacity) and at the end of expiration (near functional residual capacity). All subjects were coached in the breath holding techniques, and practised breath holding, immediately prior to scanning. All subjects were scanned within 60 minutes of receiving 400 micrograms of salbutamol via a spacer. Images were reconstructed

with a slice thickness of 0·75 mm at a 0·5 mm interval using B35f kernel. Post processing was performed on semi-automated software, Apollo (VIDA Diagnostics, Iowa).

QCT parameters obtained included; morphometry, measured in mm^2, Lumen Area (LA), Total Area (TA), Wall Area (WA) $(TA - LA)$ and percentage Wall Area (%WA) $\left(100\ x\left(\frac{(TA-LA)}{TA}\right)\right)$. Air-trapping measures were Mean Lung Density Expiratory to Inspiratory ratio ($MLD_{E/I}$) measured in Hounsfield Units (HU). Density at 15^{th} percentile point (Perc15) was measured in HU. All morphometry measures were corrected for Body Surface Area mm^2/m^2 (BSA) $\left(\sqrt{\frac{height\ (cm)x\ weight\ (kg)}{3600}}\right)$.

4.3.7. Randomisation and masking

Randomisation was performed by the trial pharmacist using previously generated treatment allocation cards, and was stratified by whether or not participants were receiving treatment with regular oral corticosteroids, and whether they were undergoing bronchoscopy. All other site staff, patients and sponsor personnel remained blinded to treatment allocation until the study had been completed and the trial database locked. Results of sputum and blood eosinophil counts subsequent to the baseline visit were not disclosed to the investigators during the study because of the expected anti-eosinophilic effects of fevipiprant.

4.3.8. Statistical analysis

The primary outcome of the study was the change in sputum eosinophil percentage between the baseline visit and the post-treatment visit. As sputum eosinophil percentage is known to follow a log-normal distribution, the analysis was based on a log_{10}-transformed scale with results back-transformed to obtain the within-group ratios of geometric means at the end of treatment compared to baseline. We report the reciprocal of these ratios as fold-reductions from baseline within each group, and the ratio of these ratios as a measure of how many times greater the reduction in the

fevipiprant group was compared to the reduction in the placebo group. The secondary outcome was the change from baseline to post-treatment with respect to ACQ-7 score. Exploratory outcomes included the change from baseline to post-treatment with respect to ACQ-7 score in the subgroup with baseline score ≥ 1·5, AQLQ(S) score, FEV_1 and submucosal eosinophil count on bronchial biopsy. Statistical analyses were performed using SAS/STAT software, versions 9·3 and 9·4 of the SAS System for AIX (SAS Institute Inc., Cary, NC, USA) and Prism 6 (GraphPad, La Jolla, CA, USA). Changes in efficacy outcomes from the baseline to post-treatment visits were analysed using an analysis of covariance (ANCOVA) model, with treatment as the fixed effect. Randomisation strata and baseline values of efficacy variables were entered as factors in the ANCOVA model for analysis of the primary outcome, secondary outcome and exploratory outcomes. Exploratory endpoints not explicitly detailed in the statistical analysis plan were analysed without correction for randomisation stratum or baseline values. Efficacy outcomes were analysed by intention to treat and safety outcomes were analysed by treatment received. One patient was assigned to fevipiprant but incorrectly dispensed placebo at the mid-treatment visit. One patient was assigned to fevipiprant but incorrectly dispensed placebo throughout the course of the study. They were included in the fevipiprant group for efficacy analyses, but the latter patient was included in the placebo group for safety analyses. The planned sample size of 60 randomised patients was calculated so that at least 24 patients per arm would complete the post-treatment assessment in order to ensure 80% power at the two-sided 5% significance, assuming a 50% reduction in sputum eosinophil percentage with fevipiprant.

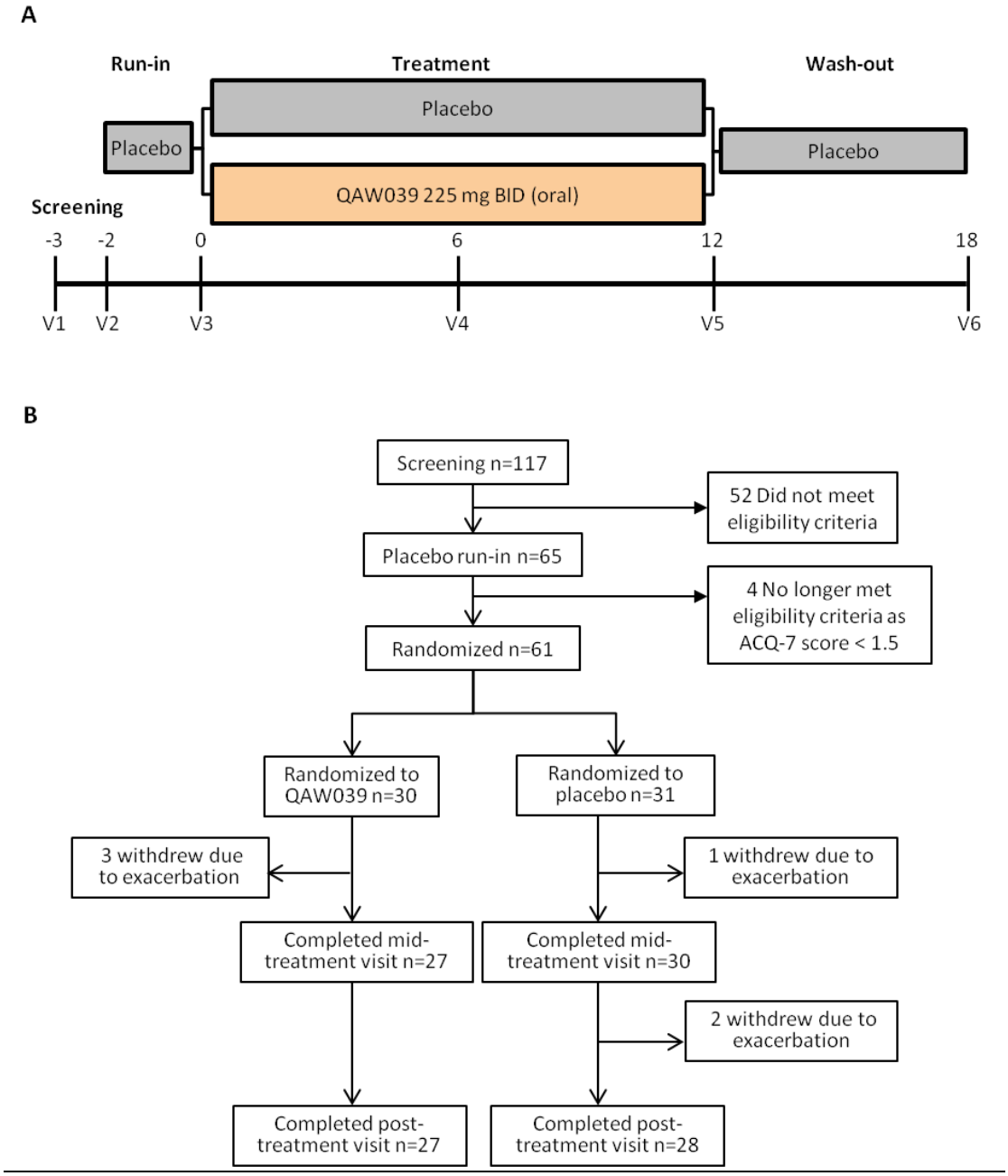

Figure 4.1: Summary of study protocol and participant flow

Panel A shows the timings of study visits and treatment allocations. Panel B shows the number of patients who attended screening, were randomised, and completed each of the study visits.

4.4. Results

4.4.1. Baseline characteristics and demographics

Participants were recruited between Feb 10, 2012 and Jan 30, 2013. A total of 117 patients attended a screening visit, of which 61 fulfilled the inclusion and exclusion criteria and were randomised (Figure 4.1b). Thirty-one patients were assigned to receive placebo and 30 to receive fevipiprant. Four patients withdrew in the placebo group and three patients in the fevipiprant group, in each case due to an exacerbation of asthma. The randomised groups were well-matched for baseline characteristics, as shown in Table 4.1.

4.4.2. Primary outcome: Sputum eosinophils

Efficacy outcomes are shown in Figures 4.2, 4.3 and 4.4, and in Tables 4.2, 4.3 and 4.4. The geometric mean sputum eosinophil percentage fell from 5·4% at baseline to 1·1% post-treatment in the fevipiprant group, and from 4·7% at baseline to 3·9% post-treatment in the placebo group. The ratio of geometric means post-treatment to baseline for the sputum eosinophil percentage was 0·78 (1·3-fold reduction) in the placebo group and 0·22 (4·5-fold reduction) in the fevipiprant group, with a 3·5-fold (95% confidence interval [CI] 1·7 to 7·0-fold) greater reduction in the fevipiprant group compared to placebo (p = 0·0014).

4.4.3. Asthma control, asthma quality of life and FEV_1

The mean ACQ-7 score fell by 0·32 points from baseline to post-treatment in the fevipiprant group compared to the change seen with placebo, but this improvement did not reach statistical significance (95% CI -0·78, 0·14; p = 0·17). However, among the subset of patients (n = 40) uncontrolled at baseline (ACQ-7 score ≥ 1·5), the mean ACQ-7 score fell by 0·56 points compared to placebo, which was both clinically and statistically significant (95% CI -1·12, -0·01; p = 0·046). The mean AQLQ(S) score improved by 0·59 points in the fevipiprant group compared to placebo, which was statistically significant (95% CI 0·16, 1·03; p = 0·0080). The mean post-bronchodilator

FEV_1 increased by 0·16L from baseline to post-treatment in the fevipiprant group compared to placebo, with a statistically significant difference between the groups (95% CI 0·03, 0·30; p = 0·021). There were no significant differences between the groups with respect to changes in pre-bronchodilator FEV_1. There were no significant changes in peripheral blood eosinophil count or exhaled nitric oxide in either group.

4.4.4. Microscopic airway inflammation and airway remodelling

Paired bronchial biopsies (baseline and post-treatment) were obtained in 14 patients in the fevipiprant group and 12 patients in the placebo group. We observed a 2·5-fold greater reduction in bronchial submucosal eosinophil numbers from baseline to post-treatment in the fevipiprant group compared to the placebo group (p = 0·040). There was a 1·4-fold reduction in bronchial epithelial eosinophil numbers from baseline in favour of fevipiprant, but the treatment difference did not reach statistical significance. A 2.1µm reduction in reticular basement membrane thickness (RBM) was observed in the treatment group compared to placebo but, similarly, this difference was not statistically significant. Subjects treated with fevipiprant demonstrated a 27·8 percentage point increase in the proportion of intact epithelium (95% CI 2·9, 52·7; p = 0·030), and a 26·6 percentage point reduction in the proportion of denuded epithelium (95% CI -44·9, -8·3; p = 0·0062), compared to the change seen with placebo. Changes in epithelial integrity were not significantly correlated with changes in sputum or bronchial mucosal eosinophilic inflammation, as shown in Figure 4.6. Patients treated with fevipiprant showed 15.2 percentage point reduction in ASM% compared to change seen in placebo (95% CI -1.4, -31.1; p = 0·034). See Figure 4.5.

4.4.5. Computed tomography, safety and other outcomes:

Functional residual capacity (FRC) fell by 0·31 L in the fevipiprant group compared to the change seen with placebo (95% CI -0·62, -0·001; p = 0·049) and expiratory CT lung volume fell by 216 cm^3 in the fevipiprant group compared to the placebo group (95%

CI -391, -40; p = 0·017), but no significant treatment differences were observed with respect to other quantitative CT parameters. Significant positive correlations were observed between changes in plethysmographic and CT-derived measures of expiratory air trapping, as shown in Figure 4.7.

Outcomes measured following the 6 week washout period returned to baseline without any significant differences between baseline and post-washout for any outcome. Fevipiprant had an acceptable side-effect profile throughout the study period. Total adverse events and adverse events within each organ class were balanced between the two treatment groups. There were no deaths or serious adverse events reported, and no patient withdrawals suspected by the investigator to be related to the study drug, as shown in Table 4.5.

Table 4.1: Baseline Characteristics of Randomised Population

Characteristic	**Fevipiprant (n = 30)**	**Placebo (n = 31)**
***Sex** (no. of subjects):* Male	18	13
Female	12	18
***Age** (yr):* Mean	50	50
Range	20 – 80	19 – 68
Duration of asthma (yr)	32 ± 16	29 ± 15
Body-mass index (kg/m^2)	31·0 ± 5·9	29·6 ± 6·0
Positive atopic status:(% of subjects)‡	87	84
Number of exacerbations in previous year	1.8 ± 1.7	2.2 ± 2.8
Number of patients (%) with rhinosinusitis	12/30 (40.0)	11/31 (35.5)
Number of patients (%) with nasal polyps	5/30 (16.7)	3/31 (9.7)
Total IgE (U/ml): Median	414	388
Interquartile range	216 – 863	181 – 1121
Pre-bronchodilator FEV_1 (% of predicted value)	72·5 ± 23·8	75·1 ± 27·3
Pre-bronchodilator FEV_1/FVC (%)		
Median	68·0	69·2
Interquartile range	46·7 – 73·6	52·1 – 73·5
Improvement in Pre-bronchodilator FEV_1 (%)		
Median	9·3	12·0
Interquartile range	5·5 – 12·6	6·1 – 29·9
Eosinophil count in sputum (%) ¶	5·31 (2·77)	4·24 (4·03)
Eosinophil count in blood (×10^9/L) ¶	0·28 (1·31)	0·28 (0·79)
$FENO_{50}$ (ppb)	30 ± 24	48 ± 43
Score on Asthma Control Questionnaire	1·9 ± 0·8	2·2 ± 0·9
Score on Asthma Quality of Life Questionnaire	5·4 ± 1·1	5·0 ± 1·0
Inhaled corticosteroid dose (beclomethasone dipropionate equivalent [µg])		
Median	1600	1000
Interquartile range	800 – 1600	800 – 1600
Number of patients (%) using long-acting beta-agonists	27/30 (90)	26/31 (84)
Number of patients (%) using regular oral prednisolone	7/30 (23)	7/31 (23)
Global Initiative for Asthma treatment step (number of patients)*		
Step 2	1	1
Step 3	1	4
Step 4	21	19
Step 5	7	7

Plus-minus values are means ± standard deviation (SD) unless otherwise stated.

‡ Positive atopic status was defined as a positive skin test for any of a panel of specified aeroallergens grass pollen, tree pollen, moulds, cat fur, dog dander, and house dust mite

¶ Expressed as geometric mean (coefficient of variation) Global Initiative for Asthma treatment steps.*

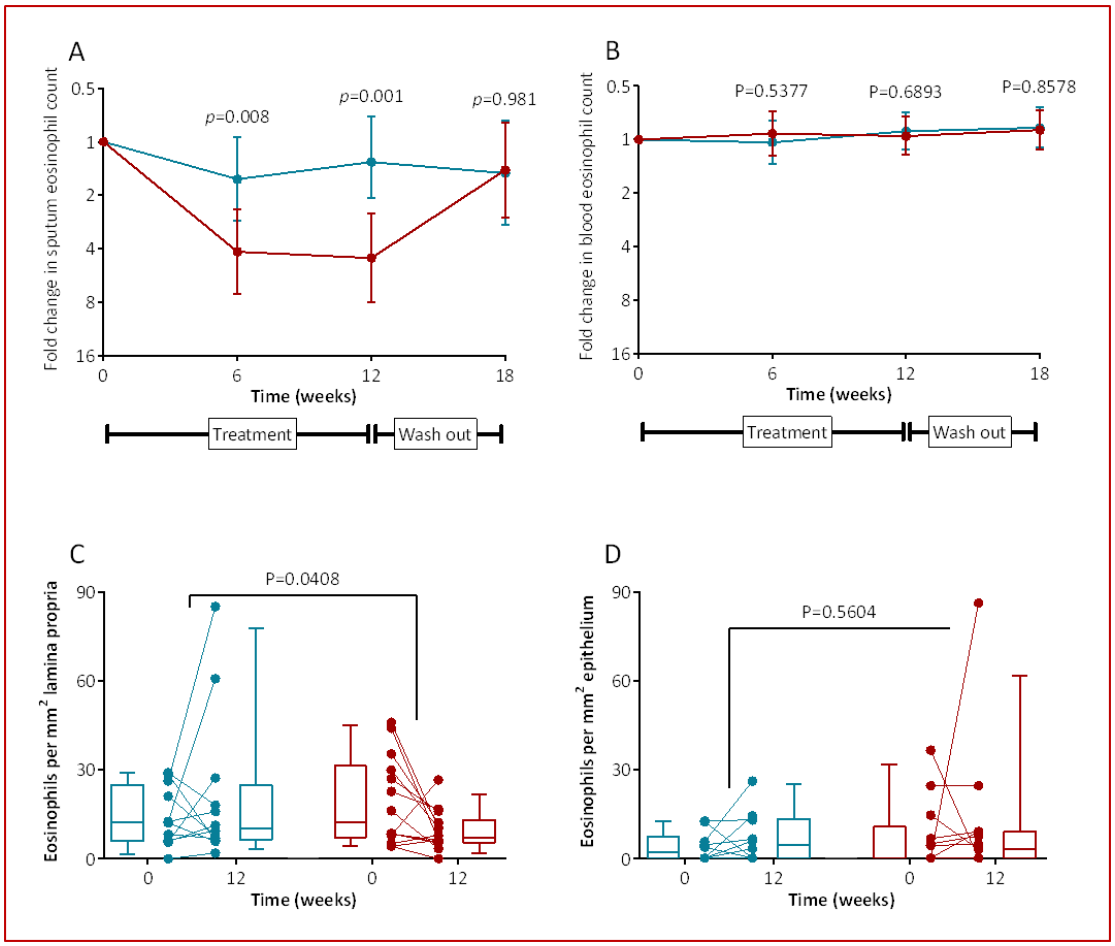

Figure 4.2: Comparison of eosinophilic inflammation outcomes between the study groups

Panels A and B show fold-reductions in sputum and blood eosinophil counts respectively at each study visit compared to the baseline visit, in the placebo (blue square) and fevipiprant (orange circle) groups. P values refer to differences between the study groups with respect to change from the baseline visit. Panels C and D show lamina propria and epithelial eosinophil numbers respectively at the baseline and post-treatment visits, in the placebo (blue square) and fevipiprant (orange circle) groups. Box and whisker plots show the median, 25th and 75th percentiles as a box, and the 10th and 90th percentiles as whiskers. P values refer to differences between the study groups with respect to change from the baseline visit to the post-treatment visit.

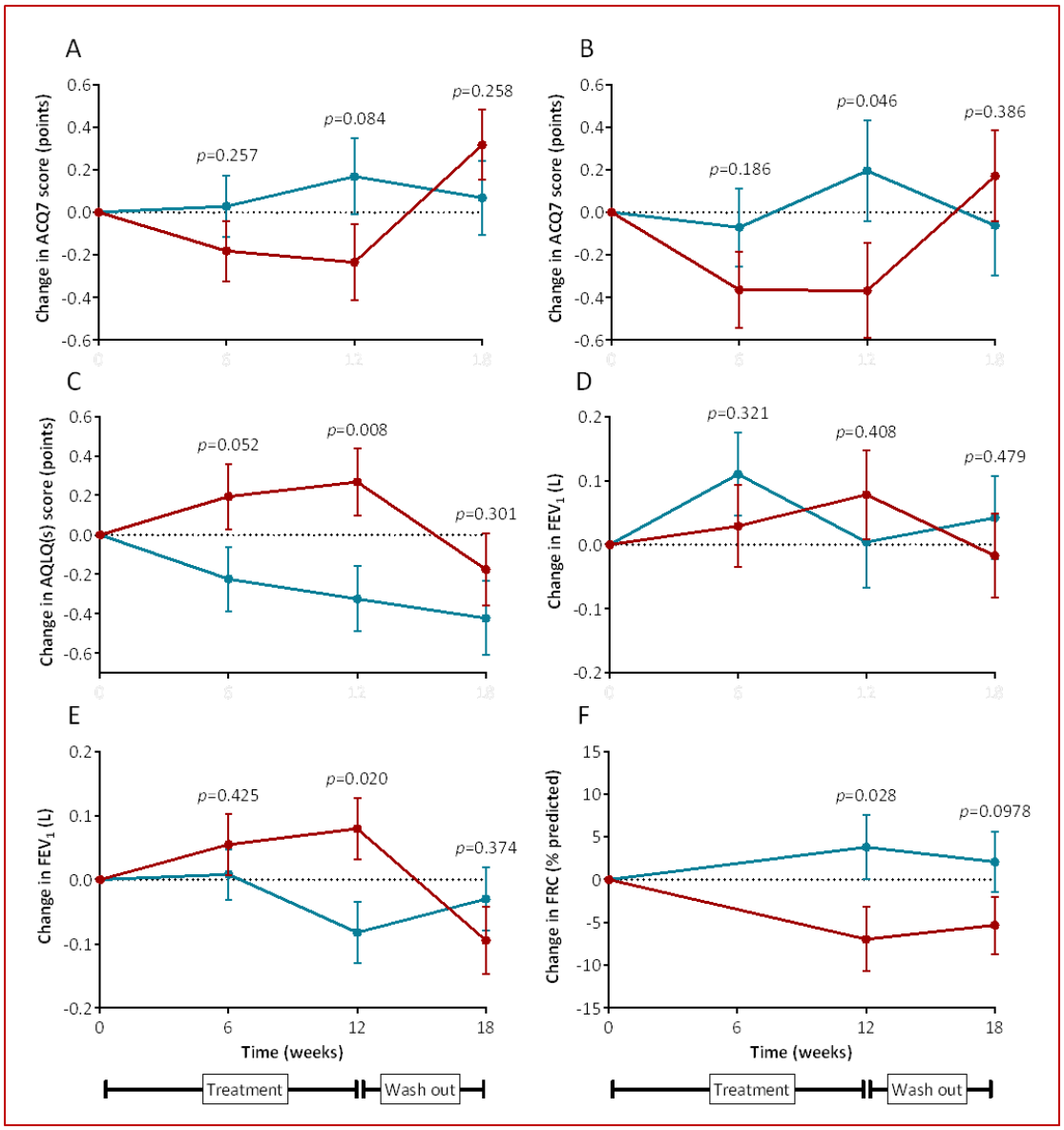

Figure 4.3: Comparison of patient-reported and lung function outcome measures between the study groups:

Changes compared to the baseline visit are shown in the placebo (blue square) and fevipiprant (orange circle) groups with respect to ACQ7 (Panel A), ACQ7 in the subgroup with a baseline value ≥ 1·5 (Panel B), AQLQ(S) (Panel C), pre-bronchodilator FEV_1 (Panel D), post-bronchodilator FEV_1 (Panel E), and functional residual capacity (FRC, Panel F). P values refer to differences between the study groups with respect to change from the baseline visit.

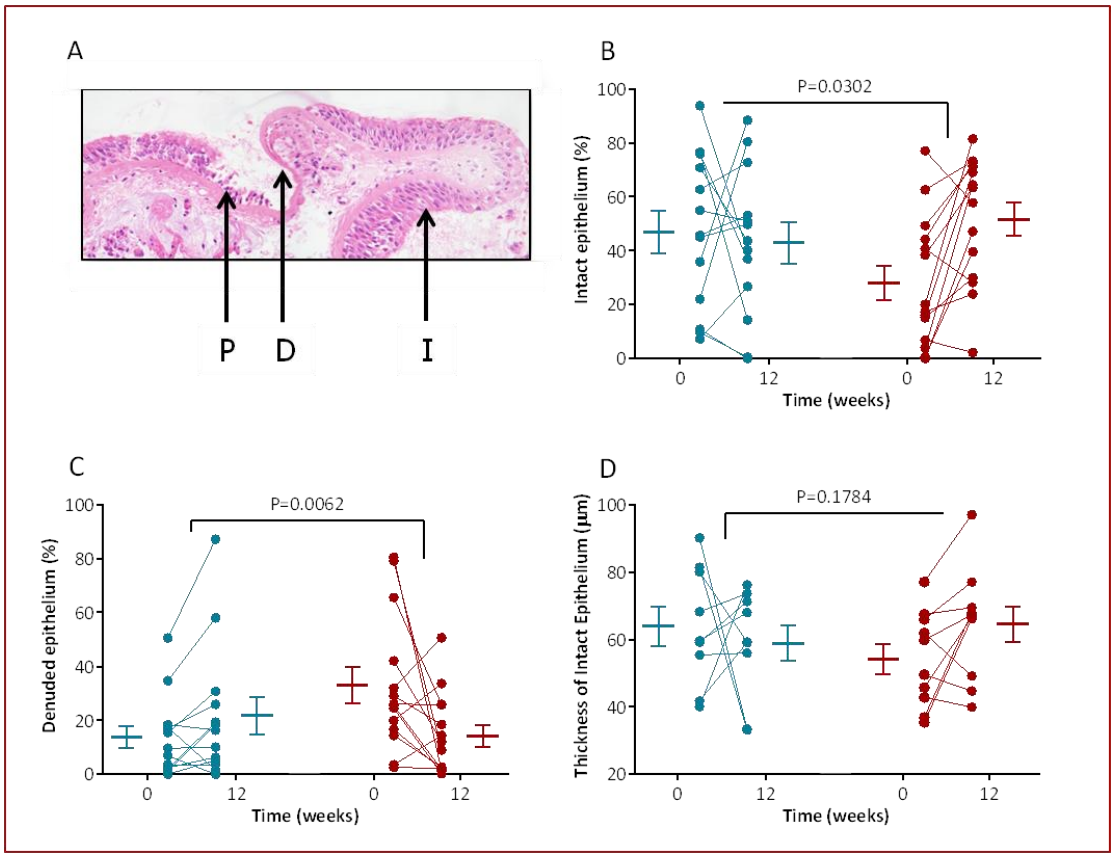

Figure 4.4: Comparison of epithelial damage outcome measures between the study groups

Panel A shows a photomicrograph of a bronchial biopsy specimen demonstrating the appearance of intact epithelium (I), partially denuded epithelium (P) and denuded epithelium (D). Panels B-D show percentage of epithelium that is intact, percentage of epithelium that is denuded and thickness of intact epithelium respectively at the baseline and post-treatment visits, in the placebo (blue square) and fevipiprant (orange circle) groups. Error bars indicate the mean plus or minus the standard error of the mean. P values refer to differences between the study groups with respect to change from the baseline visit to the post-treatment visit.

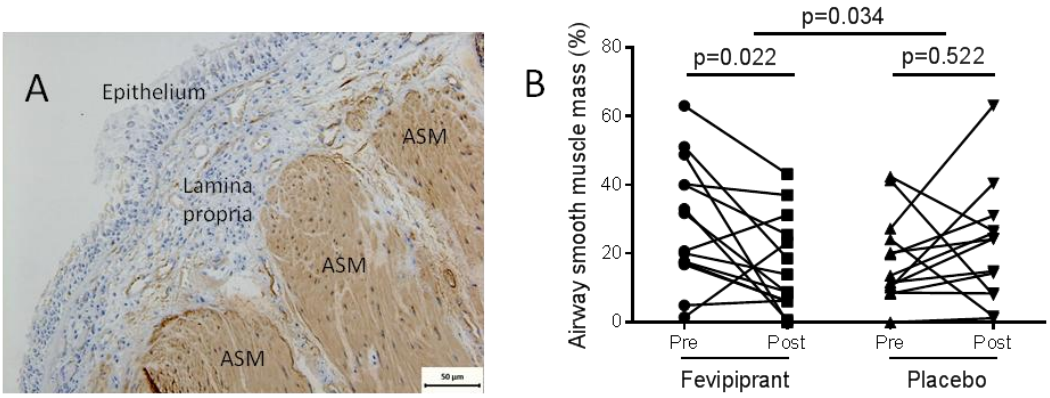

Figure 4.5: The effect of fevipiprant on ASM remodelling.

A: Photomicrograph of a bronchial biopsy showing increased airway smooth muscle mass (brown stained *alpha*-smooth muscle actin), epithelium and lamina propria.

B: Airway smooth muscle mass, as measured by % *alpha*-smooth muscle actin positive area, in bronchial biopsies in subjects treated with fevipiprant and placebo.

Table 4.2: Outcome Measures at Baseline and Post-Treatment in the Full Analysis Set Population

Outcome	Baseline values		Post-treatment values		Change from baseline to post-treatment			
	Fevipiprant	Placebo	Fevipiprant	Placebo	Fevipiprant (N = 30)	Placebo (N = 31)	Treatment difference (Fevipiprant vs placebo)	P value
Eosinophil count in sputum (%) ‡	5·42 (287·65)	4·65 (391·44)	1·12 (0·65, 1·93)	3·88 (2·26, 6·67)	0·22 (0·13, 0·39)	0·78 (0·45, 1·33)	0·29 (0·14, 0·58)	0·0014
Eosinophil count in blood ($\times 10^9$/L) ‡	0·29 (95·03)	0·28 (80·63)	0·29 (0·23, 0·36)	0·32 (0·25, 0·41)	1·01 (0·79, 1·28)	1·13 (0·89, 1·43)	0·89 (0·66, 1·20)	0·44
$FENO_{50}$ (ppb)	37·72 (4·75)	43·67 (6·97)	34·88 (3·97)	38·48 (4·32)	-5·82 (-13·79, 2·16)	-2·21 (-10·90, 6·48)	-3·60 (-13·93, 6·72)	0·49
ACQ-7 score	1·91 (0·15)	2·22 (0·16)	1·89 (0·18)	2·21 (0·18)	-0·18 (0·18)	0·14 (0·18)	-0·32 (-0·78, 0·14)	0·17
ACQ-7 score in subjects with baseline ≥1·5†	2·37 (0·11)	2·57 (0·15)	1·69 (0·22)	2·25 (0·23)	-0·37 (0·22)	0·20 (0·23)	-0·56 (-1·12, -0·01)	0·046
ACQ-6 score	1·71 (0·18)	2·11 (0·17)	1·66 (0·19)	2·11 (0·19)	-0·26 (-0·65, 0·13)	0·19 (-0·20, 0·59)	-0·45 (-0·96, 0·05)	0·077
AQLQ score Total	5·43 (0·20)	5·02 (0·18)	5·48 (0·17)	4·89 (0·17)	0·27 (-0·07, 0·61)	-0·33 (-0·66, 0·01)	0·59 (0·16, 1·03)	0·0080
AQLQ score Symptoms	5·22 (0·21)	4·73 (0·20)	5·25 (0·22)	4·62 (0·21)	0·28 (-0·15, 0·72)	-0·34 (-0·77, 0·09)	0·63 (0·07, 1·18)	0·028
AQLQ score Activities	5·58 (0·20)	5·28 (0·20)	5·70 (0·15)	5·16 (0·15)	0·28 (-0·03, 0·59)	-0·26 (-0·57, 0·05)	0·54 (0·14, 0·93)	0·0087

Outcome	Baseline values		Post-treatment values		Change from baseline to post-treatment			
	Fevipiprant	Placebo	Fevipiprant	Placebo	Fevipiprant (N = 30)	Placebo (N = 31)	Treatment difference (Fevipiprant vs placebo)	P value
AQLQ score Emotions	5·48 (0·26)	4·90 (0·21)	5·50 (0·22)	4·65 (0·21)	0·33 (-0·10, 0·76)	-0·53 (-0·95, -0·11)	0·86 (0·31, 1·40)	0·0027
AQLQ score Environmental	5·62 (0·27)	5·28 (0·23)	5·74 (0·19)	5·19 (0·18)	0·30 (-0·08, 0·67)	-0·25 (-0·62, 0·12)	0·55 (0·07, 1·02)	0·025
Pre-bronchodilator FEV_1 (L)	2·27 (0·17)	2·27 (0·18)	2·35 (0·07)	2·28 (0·07)	0·08 (-0·06, 0·22)	0·004 (-0·14, 0·15)	0·07 (-0·10, 0·25)	0·41
Pre-bronchodilator FEV_1/FVC (%)	63·67 (2·85)	64·23 (2·17)	65·49 (0·88)	63·61 (0·88)	1·54 (-0·22, 3·29)	-0·34 (-2·12, 1·43)	1·88 (-0·38, 4·14)	0·10
Post-bronchodilator FEV_1 (L)	2·49 (0·17)	2·71 (0·19)	2·66 (0·05)	2·50 (0·05)	0·06 (-0·05, 0·17)	-0·10 (-0·21, 0·01)	0·16 (0·03, 0·30)	0·021
Post-bronchodilator FEV_1/FVC (%)	66·90 (2·82)	69·72 (2·14)	70·42 (1·02)	67·31 (1·03)	2·10 (0·05, 4·15)	-1·00 (-3·07, 1·06)	3·11 (0·46, 5·75)	0·023
RV (L)	2·78 (0·22)	2·87 (0·23)	2·66 (0·13)	2·79 (0·12)	-0·17 (-0·42, 0·09)	-0·03 (-0·29, 0·22)	-0·14 (-0·46, 0·19)	0·40
TLC (L)	6·49 (0·29)	6·41 (0·29)	6·40 (0·13)	6·37 (0·13)	-0·04 (-0·30, 0·21)	-0·08 (-0·33, 0·18)	0·04 (-0·29, 0·36)	0·83
RV/TLC (%)	42·28 (2·30)	44·29 (2·49)	40·70 (1·30)	43·08 (1·32)	-2·64 (-5·24, -0·04)	-0·260 (-2·92, 2·40)	-2·38 (-5·69, 0·93)	0·15
FRC (L)	3·90 (0·26)	3·73 (0·24)	3·58 (0·12)	3·88 (0·12)	-0·23 (-0·48, 0·01)	0·08 (-0·17, 0·32)	-0·31 (-0·62,-0·001)	0·049
KCO (% predicted)	108·93 (4·39)	104·87 (3·28)	106·47 (1·72)	109·01 (1·70)	-0·32 (-3·78, 3·14)	2·22 (-1·20, 5·63)	-2·54 (-6·90, 1·83)	0·25

$FE_{NO_{50}}$ denotes the fraction of exhaled nitric oxide in exhaled air at a flow rate of 50 ml/s, ACQ-7 seven-point Asthma Control Questionnaire score, ACQ-6 six-point Asthma Control Questionnaire score (not including spirometry contribution), AQLQ Asthma Quality of Life Questionnaire score, FEV_1 forced expiratory volume in one second, FVC forced vital capacity, RV residual volume, TLC total lung capacity, FRC functional residual capacity, and K_{CO} carbon monoxide transfer coefficient.

Baseline and post-treatment values are mean (standard error), change from baseline to post-treatment is mean change (lower limit, upper limit of 95% confidence interval), and treatment difference is mean change in fevipiprant group minus mean change in placebo group (lower limit, upper limit of 95% confidence interval), unless otherwise stated. Post-treatment and changes from baseline to post-treatment are covariate-adjusted (least square mean) values.

‡ Baseline values are geometric mean (% coefficient of variation), post-treatment values are geometric mean (lower limit, upper limit of 95% confidence interval), change from baseline to post-treatment is geometric mean fold-change (lower limit, upper limit of 95% confidence interval), and treatment difference is ratio of geometric mean fold-change in fevipiprant group to geometric mean fold-change in placebo group (lower limit, upper limit of 95% confidence interval).

†N = 18 in fevipiprant group and N = 22 in placebo group.

Table 4.3: Bronchial biopsy outcome measures

Outcome	Baseline values		Post-treatment values		Change from baseline to post-treatment			
	Fevipiprant	Placebo	Fevipiprant	Placebo	Fevipiprant (N = 14)	Placebo (N = 12)	Treatment difference (Fevipiprant vs placebo)	P value
Inflammatory cells*								
Eosinophils/mm^2 lamina propria	13·9 (23·5)	9·1 (39·4)	6·7 (28·8)	15·8 (33·3)	0·6 (0·3, 1·0)	1·4 (0·7, 2·7)	0·4 (0·2, 1·0)	0·040
CD3+ cells/mm^2 lamina propria	9·0 (37·7)	11·1 (31·2)	14·4 (29·8)	14·6 (31·0)	1·6 (0·7, 3·9)	1·3 (0·5, 3·3)	1·2 (0·3, 4·3)	0·75
Mast cells/mm^2 lamina propria	5·5 (36·7)	8·9 (26·5)	4·7 (27·4)	11·9 (28·5)	0·9 (0·5, 1·5)	1·3 (0·8, 2·3)	0·6 (0·3, 1·4)	0·25
Neutrophils/mm^2 lamina propria	1·3 (38·5)	3·1 (36·5)	1·3 (31·8)	3·5 (33·1)	1·0 (0·4, 2·4)	1·1 (0·4, 2·8)	0·9 (0·2, 3·2)	0·84
Eosinophils/mm^2 epithelium	2·5 (45·9)	2·2 (42·8)	2·4 (50·5)	3·5 (67·3)	1·0 (0·4, 2·8)	1·5 (0·4, 5·4)	0·7 (0·2, 2·9)	0·59
CD3+ cells/mm^2 epithelium	2·0 (38·8)	3·9 (53·8)	4·1 (47·0)	3·2 (56·8)	1·7 (0·5, 5·4)	0·8 (0·2, 3·2)	2·0 (0·3, 12·0)	0·42
Mast cells/mm^2 epithelium	0·8 (21·2)	1·0 (22·7)	1·7 (32·8)	1·8 (39·2)	1·9 (1·0, 3·4)	1·8 (0·9, 3·6)	1·0 (0·4, 2·6)	0·93
Neutrophils/mm^2 epithelium	0·7 (8·6)	2·1 (53·2)	0·8 (18·7)	0·8 (22·2)	1·1 (0·6, 2·3)	0·4 (0·2, 0·8)	3·1 (1·0, 9·1)	0·044
Mast cells/mm^2 airway smooth muscle	3·4 (43·4)	4·3 (51·1)	2·9 (53·8)	4·8 (56·6)	0·7 (0·2, 1·9)	1·1 (0·4, 3·0)	0·6 (0·1, 2·6)	0·48

Outcome	Baseline values		Post-treatment values		Change from baseline to post-treatment			
	Fevipiprant	Placebo	Fevipiprant	Placebo	Fevipiprant (N = 14)	Placebo (N = 12)	Treatment difference (Fevipiprant vs placebo)	P value
Tissue remodeling†								
MUC5AC cells/mm intact epithelial length	38·3 (6·7)	24·6 (7·9)	55·6 (7·6)	37·1 (7·9)	12·8 (-7·3, 32·9)	10·2 (-7·6, 27·9)	2·7 (-24·1, 29·5)	0·84
Goblet cells/mm intact epithelial length	13·7 (4·2)	11·6 (3·3)	22·3 (4·2)	23·2 (4·8)	7·9 (-0·6, 16·5)	11·6 (3·6, 19·7)	-3·7 (-15·5, 8·0)	0·51
Goblet cells/mm^2 intact epithelial area	287·8 (83·6)	209·7 (48·5)	366·9 (90·7)	457·0 (103·4)	58·9 (-147, 265·2)	247·4 (51·7, 443·0)	-188 (-473, 95·9)	0·18
Vessel score (mean Chalkley count)	5·8 (0·3)	6·6 (0·5)	5·9 (0·4)	5·8 (0·4)	0·1 (-0·8, 1·0)	-0·8 (-1·8, 0·1)	0·9 (-0·4, 2·2)	0·17
Intact epithelium (% of total length)	28·0 (6·5)	47·0 (7·9)	51·7 (6·9)	42·9 (7·1)	23·7 (6·4, 41·0)	-4·1 (-22·0, 13·8)	27·8 (2·9, 52·7)	0·030
Partially intact epithelium (% of total length)	39·0 (4·3)	39·2 (6·4)	34·0 (4·8)	35·3 (5·0)	-5·0 (-19·6, 9·5)	-3·9 (-18·9, 11·2)	-1·2 (-22·1, 19·7)	0·91
Denuded epithelium (% of total length)	33·0 (6·7)	13·8 (4·1)	14·3 (5·5)	21·7 (5·7)	-18·6 (-31·4, -5·9)	8·0 (-5·2, 21·2)	-26·6 (-44·9, -8·3)	0·006 2
Epithelial thickness (µm)	54·3 (4·5)	64·0 (5·8)	67·3 (4·3)	58·4 (4·9)	10·3 (-5·6, 26·2)	-5·1 (-21·9, 11·7)	15·4 (-7·7, 38·5)	0·18
RBM thickness (µm)	14·9 (1·2)	10·4 (1·0)	11·3 (1·1)	13·4 (1·2)	-1·5 (-3·8, 0·7)	0·6 (-1·9, 3·0)	-2·1 (-5·4, 1·2)	0·20
Airway Smooth muscle (%)	29.1 (4.8)	18.4 (3.5)	16.4 (3.7)	21.9 (4.7)	-12.7 (-23.2, -2.2)	2.5 (-8.1, 15.1)	-15.2% (-1.4, -31.1)	0.034

**Baseline and post-treatment values are geometric mean (% coefficient of variation), change from baseline to post-treatment is geometric mean fold-change (lower limit, upper limit of 95% confidence interval), and treatment difference is ratio of geometric mean fold-change in fevipiprant group to geometric mean fold-change in placebo group (lower limit, upper limit of 95% confidence interval).*

†Baseline and post-treatment values are mean (standard error), change from baseline to post-treatment is mean change (lower limit, upper limit of 95% confidence interval), and treatment difference is mean change in fevipiprant group minus mean change in placebo group (lower limit, upper limit of 95% confidence interval).

Table 4.4: Quantitative computed tomography and densitometry

Outcome	Baseline values		Post-treatment values		Change from baseline to post-treatment			
	Fevipiprant	**Placebo**	**Fevipiprant**	**Placebo**	**Fevipiprant (N = 23)**	**Placebo (N = 26)**	**Treatment difference (Fevipiprant vs placebo)**	**P value**
RB1 wall area / BSA (mm^2/m^2)	19·74 (1·09)	19·59 (0·94)	20·89 (1·50)	18·37 (1·41)	1·15 (-0·89, 3·18)	-1·23 (-3·14, 0·68)	2·38 (-0·41, 5·17)	0·093
RB1 luminal area / BSA (mm^2/m^2)	12·54 (1·45)	11·11 (0·79)	14·22 (2·05)	11·02 (1·92)	1·68 (-1·55, 4·90)	-0·09 (-3·12, 2·93)	1·77 (-2·65, 6·19)	0·42
RB1 percentage wall area (%)	62·7 (1·3)	64·5 (0·8)	63·2 (1·1)	63·1 (1·1)	0·4 (-1·3, 2·2)	-1·4 (-3·0, 0·3)	1·8 (-0·6, 4·2)	0·14
Average wall area / BSA (mm^2/m^2)	17·3 (0·5)	17·7 (0·6)	17·6 (0·6)	17·8 (0·6)	0·3 (-0·5, 1·1)	0·1 (-0·7, 0·9)	0·2 (-0·9, 1·3)	0·75
Average lumen area / BSA (mm^2/m^2)	10·6 (0·5)	10·9 (0·6)	10·7 (0·6)	11·5 (0·6)	0·2 (-0·6, 1·0)	0·6 (-0·2, 1·3)	-0·4 (-1·5, 0·7)	0·45
Average percentage wall area (%)	63·2 (0·5)	62·8 (0·4)	62·9 (0·3)	62·3 (0·3)	-0·3 (-1·0, 0·4)	-0·5 (-1·1, 0·1)	0·2 (-0·7, 1·1)	0·67
Inspiratory MLD (HU)	-829·1 (7·7)	-837·2 (6·9)	-839·7 (5·4)	-846·5 (5·1)	-10·6 (-20·7, -0·6)	-9·3 (-18·7, 0·2)	-1·4 (-15·2, 12·4)	0·84
Expiratory MLD (HU)	-704·8 (15·0)	-719·1 (10·6)	-706·6 (12·7)	-732·7 (11·7)	-1·0 (-11·5, 9·6)	-13·0 (-22·8, -3·2)	12·0 (-2·4, 26·4)	0·099
MLD E/I	0·851 (0·016)	0·861 (0·013)	0·841 (0·014)	0·865 (0·013)	-0·010 (-0·026, 0·005)	0·003 (-0·012, 0·017)	-0·013 (-0·034, 0·008)	0·22
Inspiratory VI <-950 HU (cm^3)	13·7 (1·4)	14·3 (1·2)	15·2 (1·2)	14·9 (1·2)	1·5 (0·0, 3·1)	0·7 (-0·8, 2·1)	0·8 (-1·2, 2·9)	0·42
Expiratory VI <-856 HU (cm^3)	21·4 (3·7)	22·0 (2·8)	21·8 (3·4)	24·1 (3·1)	0·4 (-2·0, 2·8)	2·2 (-0·1, 4·4)	-1·8 (-5·1, 1·5)	0·27

Outcome	Baseline values		Post-treatment values		Change from baseline to post-treatment			
	Fevipiprant	Placebo	Fevipiprant	Placebo	Fevipiprant (N = 23)	Placebo (N = 26)	Treatment difference (Fevipiprant vs placebo)	P value
CTLV expiratory (cm^3)	3040 (199)	3209 (188)	3004 (225)	3420 (206)	-10 (-138, 118)	205 (86, 325)	-216 (-391, -40)	0·017
CTLV inspiratory (cm^3)	5221 (252)	5588 (297)	5419 (266)	5809 (251)	198 (-58, 454)	222 (-19, 462)	-24 (-375, 328)	0·89
CT lung volume E/I	0·588 (0·026)	0·583 (0·030)	0·557 (0·026)	0·582 (0·024)	-0·030 (-0·066, 0·006)	-0·006 (-0·040, 0·027)	-0·024 (-0·073, 0·026)	0·34
P_{15} (HU)	-939·7 (5·3)	-942·4 (4·0)	-946·5 (3·6)	-947·2 (3·4)	-6·9 (-13·2, -0·5)	-4·8 (-10·7, 1·2)	-2·1 (-10·8, 6·6)	0·63
Pi10 (mm^2)	15·6 (0·4)	14·8 (0·2)	16·0 (0·4)	15·0 (0·4)	0·3 (-0·2, 0·9)	0·2 (-0·3, 0·8)	0·1 (-0·7, 0·9)	0·76
Po20 (%)	56·5 (0·3)	56·5 (0·4)	57·8 (0·7)	56·8 (0·7)	1·3 (0·0, 2·6)	0·4 (-0·9, 1·6)	0·9 (-0·9, 2·7)	0·32

MLD denotes mean lung density, HU Hounsfield unit, E/I expiratory/inspiratory, RB1 right upper lobe apical segmental bronchus, BSA body surface area, VI voxel index, CTLV computed tomography lung volume, P_{15} Hounsfield unit value below which 15% of voxel attenuation values fall, Pi10 wall area of a theoretical airway with an internal perimeter of 10mm, and Po20 percentage wall area of a theoretical airway with an external perimeter of 20mm. Baseline and post-treatment values are mean (standard error), change from baseline to post-treatment is mean change (lower limit, upper limit of 95% confidence interval), and treatment difference is mean change in fevipiprant group minus mean change in placebo group (lower limit, upper limit of 95% confidence interval).

Table 4.5: Summary of Adverse Events.

Group	**Period between baseline and post-treatment visits**		**Period between baseline and end-of-study visits**	
	Fevipiprant (N=29) n (%)	**Placebo (N=32)** n (%)	**Fevipiprant (N=29)** n (%)	**Placebo (N=32)** n (%)
Patients with at least 1 AE	**21 (72·4)**	**25 (78·1)**	**24 (82·8)**	**26 (81·3)**
Primary system organ class				
Infections and infestations	8 (27·6)	8 (25·0)	11 (37·9)	10 (31·3)
Respiratory, thoracic and mediastinal disorders	6 (20·7)	9 (28·1)	12 (41·4)	15 (46·9)
Gastrointestinal disorders	3 (10·3)	6 (18·8)	5 (17·2)	8 (25·0)
Nervous system disorders	3 (10·3)	6 (18·8)	3 (10·3)	8 (25·0)
Injury, poisoning and procedural complications	5 (17·2)	1 (3·1)	5 (17·2)	2 (6·3)
Musculoskeletal and connective tissue disorders	1 (3·4)	3 (9·4)	3 (10·3)	3 (9·4)
General disorders and administration site conditions	3 (10·3)	1 (3·1)	3 (10·3)	2 (6·3)
Blood and lymphatic system disorders	2 (6·9)	0	2 (6·9)	0
Eye disorders	0	2 (6·3)	0	2 (6·3)
Investigations	1 (3·4)	1 (3·1)	2 (6·9)	2 (6·3)
Metabolism and nutrition disorders	2 (6·9)	0	2 (6·9)	0
Cardiac disorders	0	1 (3·1)	0	1 (3·1)
Renal and urinary disorders	1 (3·4)	0	2 (6·9)	0
Reproductive system and breast disorders	0	1 (3·1)	0	1 (3·1)
Skin and subcutaneous tissue disorders	0	1 (3·1)	1 (3·4)	1 (3·1)
Immune system disorders	0	0	0	1 (3·1)
Surgical and medical procedures	0	0	0	1 (3·1)
Vascular disorders	0	0	0	1 (3·1)

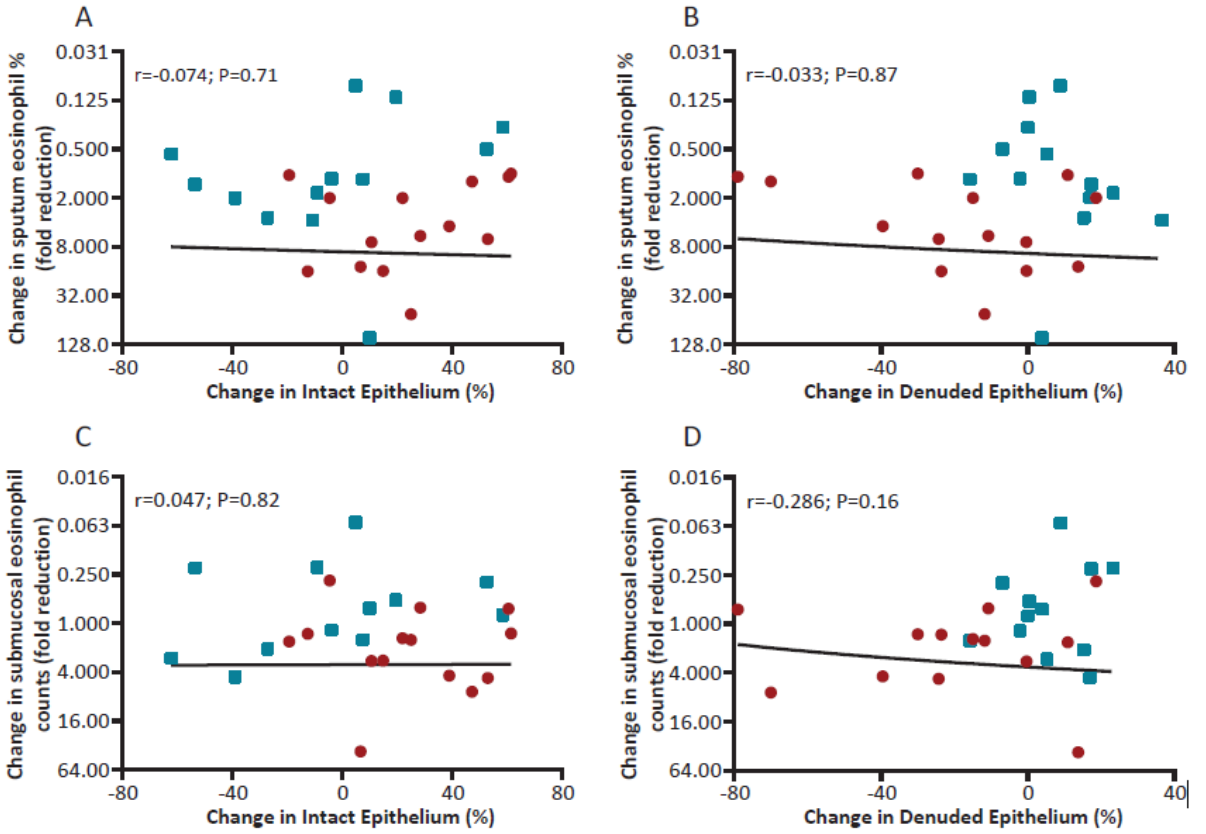

Figure 4.6: Correlations between changes in eosinophilic airway inflammation and changes in epithelial damage between the baseline and post-treatment visits

Panels A and B show correlations between fold-change in sputum eosinophil count and change in intact or denuded epithelial percentage respectively. Panels C and D show correlations between fold-change in submucosal eosinophil count and change in intact or denuded epithelial percentage respectively. Participants in the placebo and fevipiprant groups are represented by blue squares and orange circles respectively, and best-fit linear regression lines are shown for the combined group. Spearman correlation coefficients (r) and associated P values are shown.

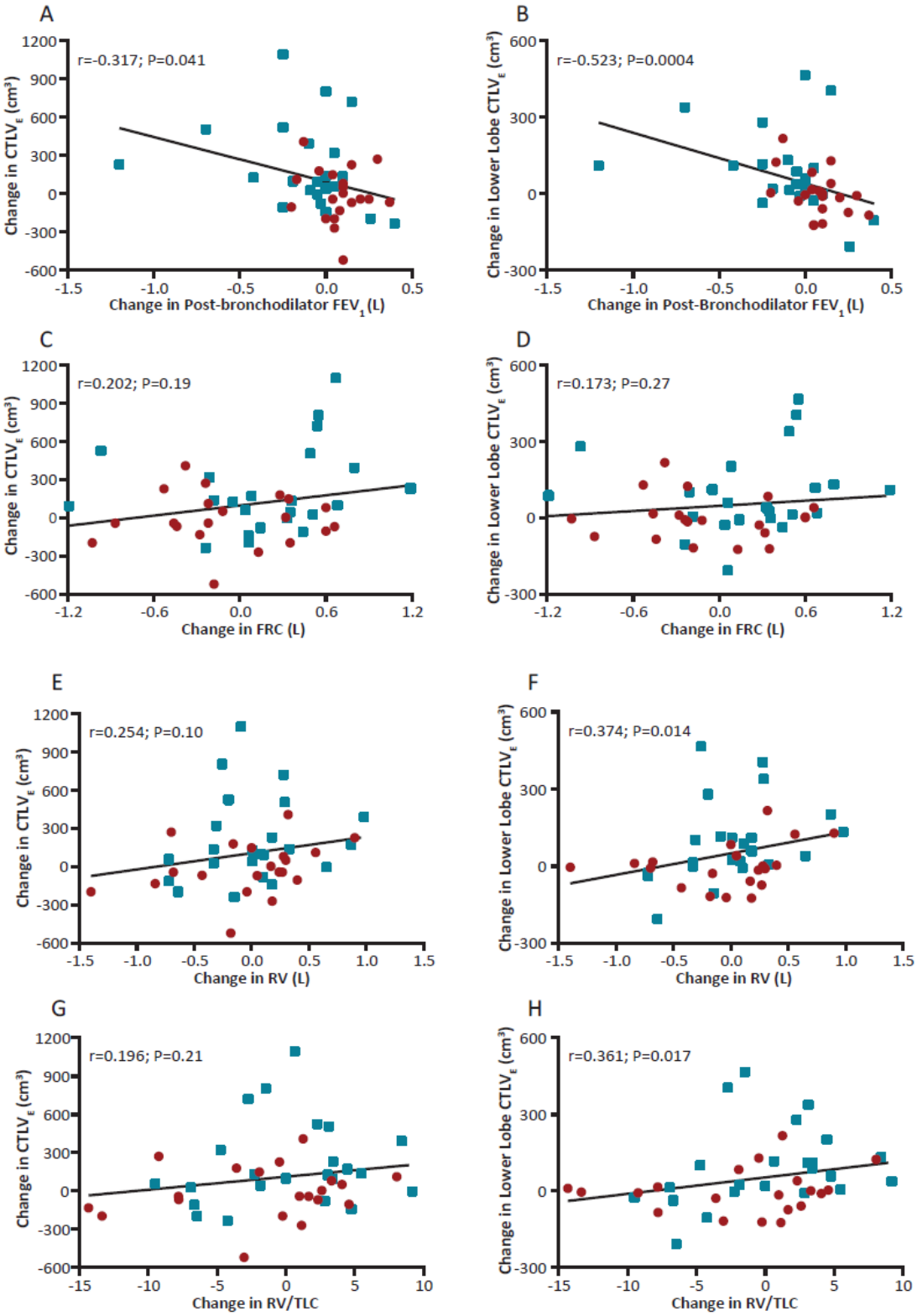

Figure 4.7: Correlations between changes in computed tomography-derived lung volumes and changes in lung function outcomes between the baseline and post-treatment visits. Correlations are shown between changes in expiratory computed

tomography-derived lung volumes ($CTLV_E$) in the whole lung or specifically the lower lobes, and changes in post-bronchodilator forced expiratory volume in one second (FEV_1, Panels A and B), functional residual capacity (FRC, Panels C and D), residual volume (RV, Panels E and F), and the ratio of residual volume to total lung capacity (RV/TLC, Panels G and H). Participants in the placebo and fevipiprant groups are represented by blue squares and orange circles respectively, and best-fit linear regression lines are shown for the combined group. Spearman correlation coefficients (r) and associated P values are shown.

4.5. Discussion

This study demonstrated that fevipiprant significantly reduced eosinophilic inflammation in the sputum and bronchial submucosa compared to placebo in patients with persistent, moderate-to-severe asthma and sputum eosinophilia. Fevipiprant significantly improved AQLQ(S) scores, post-bronchodilator FEV_1 and functional residual capacity compared to placebo in all patients, and ACQ-7 scores in the sub-group of patients who had poor asthma control at baseline (ACQ-7 ≥ 1·5 points). Exploratory analyses of bronchial biopsies suggested that fevipiprant led to improvements in epithelial integrity, ASM mass, but did not affect epithelial goblet cell number or MUC5A expression.

The magnitude of reduction in eosinophilic inflammation reported here was comparable to that observed with mepolizumab [227,229]. Unlike mepolizumab [227,229], and other anti-IL5(R) targeted biologics reslizumab and benralizumab, fevipiprant did not have any significant effect on the blood eosinophil count. This suggests that DP_2 receptor blockade attenuates the migration of eosinophils into the airway tissues, but is unlikely to have a substantial effect upon release from the bone marrow although it might exert a small indirect effect through a reduction in circulating IL-5 [324,326]. Previous interventional studies have shown that anti-eosinophilic treatments or strategies exert their major therapeutic effect through the reduction in asthma exacerbations [227,229,243,322,323], although effects on FEV_1 have also been observed, particularly in patients with blood eosinophilia [239,243]. The treatment period in this study was not long enough to observe a significant effect on exacerbations. Whether fevipiprant reduces the frequency of exacerbations in patients with eosinophilic asthma is an important question for future studies.

We noted a prompt return to baseline values following a six-week placebo wash-out period in the fevipiprant group with respect to sputum eosinophil percentage, ACQ-7 and AQLQ(S) scores, and post-bronchodilator FEV_1. There were no statistically

significant differences between baseline values and those recorded following the placebo wash-out. This suggests that the short-term improvements in asthma quality of life and post-bronchodilator FEV_1 seen with fevipiprant were driven by reversible processes rather than underlying disease modification.

We observed significant improvements in epithelial integrity following 12 weeks of treatment with fevipiprant compared to placebo. Whether this effect was a consequence of reduced eosinophilic inflammation which is known to cause epithelial damage or a direct effect upon epithelial repair and differentiation as observed *in vitro* [277] remains uncertain, although the lack of an association between changes in sputum eosinophil counts and epithelial integrity in response to fevipiprant favours a direct mechanistic effect upon the epithelium. The finding of significant reduction in ASM mass associated with fevipiprant is intriguing. Similar to epithelial improvement, there was no correlation between changes in sputum eosinophil counts and change in ASM%. This suggests possible role for PGD_2/DP_2 in ASM remodelling in asthma although this remains just a speculation and needs further studies to explore this further. This was clinically but not statistically significant reduction in RBM thickness associated with fevipiprant. This effect, unlike ASM reduction, can be explained by the anti-eosinophilic effect of fevipiprant.

Previous clinical trials of DP_2 receptor antagonists in asthma have yielded mixed results. The compound OC000459 was found to improve pre-bronchodilator FEV_1 and asthma quality of life in steroid-free patients [280], with a subsequent study finding that the beneficial effect was confined to patients with a baseline peripheral blood eosinophil count >250/µl [278]. However, this compound has not yet been tested in patients with moderate-to-severe asthma. AMG853, a dual DP_1 and DP_2 antagonist, was not effective in improving asthma symptoms or either pre- or post-bronchodilator FEV_1 in patients with moderate-to-severe asthma [334], but there is evidence that DP_1 and DP_2 stimulation may have opposing effects on a number of inflammatory mechanisms [335]. The efficacy of BI671800 was evaluated in two

separate randomised controlled trials, one in steroid-naïve adults with asthma, and one in patients receiving inhaled fluticasone [281]. In both cases, six weeks of treatment resulted in modest but statistically significant improvements in pre-bronchodilator FEV_1 compared to placebo. In these previous studies patient selection was not based upon evidence of eosinophilic airway inflammation. Previous experience has shown that targeting anti-eosinophilic therapies to patients with evidence of uncontrolled type 2 inflammation is associated with more clear evidence of efficacy [227,229,239,243,323], and the positive results obtained in our study should therefore not be extrapolated to an unselected group of patients with moderate-to-severe asthma.

One limitation of our study is the relatively small sample size undertaken in a single centre. However, the effect size in our primary outcome the sputum eosinophil count was large and other positive clinical outcomes showed both statistically and clinically important differences between the fevipiprant and placebo groups. Furthermore, our study design allowed a significant loss of efficacy to be demonstrated when fevipiprant was stopped. In contrast to many clinical trials the clinical outcomes in the group that received placebo were typically worse following intervention compared to their baseline, suggesting deterioration in this group. The lack of a positive placebo effect in this study may be explained by the fact that many of the participants were drawn from a tertiary refractory asthma clinic, and their treatment had previously been fully optimised. We also included a two-week single-blind placebo run-in period prior to the baseline visit specifically in order to minimise the placebo effect. Finally, our inclusion and exclusion criteria mandated a six-week period of clinical stability before patients could participate in the study, thus minimising the potential for changes to occur as a result of regression to the mean. Baseline characteristics of the groups were in general well-matched, although the median inhaled corticosteroid dose was numerically higher in the fevipiprant group than the placebo group. However, since background treatment remained stable throughout the study it is unlikely that this would have caused a systematic bias in

the efficacy outcome measures. During the study two dispensing errors occurred, with one patient randomised to fevipiprant and receiving placebo throughout, and a second randomised to fevipiprant and receiving placebo in the second half of the treatment period. Since efficacy outcomes were analysed by intention to treat, this could have increased the chance of a type II error. However, when efficacy outcomes were analysed by treatment received there were no significant changes in the results obtained (data not shown).

We conclude that the DP_2 receptor antagonist fevipiprant is effective at attenuating eosinophilic airway inflammation in patients with persistent eosinophilic asthma, and appears to have a favourable safety profile over a 12-week treatment period. There is evidence that fevipiprant improves lung function and asthma-related quality of life, as well as expiratory air trapping, epithelial integrity and ASM remodelling. Longer-term multi-centre studies are required to confirm these findings and to investigate the effect of fevipiprant on asthma exacerbations. Furthermore, additional *in vitro* and *in vivo* studies are needed to clarify the role of PGD_2/DP_2 axis in epithelial and ASM remodelling in asthma pathogenesis.

5. CHAPTER FIVE*:* AN OBSERVATIONAL MULTICENTRE STUDY EXAMINING AIRWAY REMODELLING AND REPAIR IN PATIENTS WITH SEVERE PERSISTENT ASTHMA TREATED WITH BRONCHIAL THERMOPLASTY, AN IMAGING AND IMMUNOPATHOLOGICAL STUDY

5.1. Abstract

Introduction: The poor understanding of structural airway remodelling in asthma remains a major obstacle in the development of new asthma therapies which are desperately needed especially for patients with severe disease. Bronchial thermoplasty (BT) is a novel asthma treatment that is claimed to target airway remodelling, however, the exact effects of BT upon airway structures is unknown. This study aimed to examine the immunopathological and imaging dynamics of airway injury and repair following bronchial thermoplasty.

Methods: This was a multicentre observational prospective study on patients assigned to have BT as part of their asthma treatment. The following was assessed pre- and post BT: asthma control, asthma quality of life, spirometry, and quantitative computed tomography. Furthermore, airway remodelling was measured on bronchial biopsies obtained at the three BT sessions. The study was registered with ISRCTN (http://www.isrctn.com/ISRCTN94263922).

Results: There was no change in spirometry, asthma symptoms questionnaire scores or QCT-derived indices of morphometry and air trapping. Biopsy analysis has shown no change in airway smooth muscle mass, however, there was reduction in actin staining areas when expressed as actin area/ASM(%) and actin area/total area(%). Epithelial integrity improved post BT with significant increase in intact epithelium and reduction of denuded epithelium.

Conclusion: Bronchial thermoplasty improves epithelial integrity and reduces actin expression possibly due to denaturation. The effect of this on various asthma domains including symptoms and exacerbations needs further research.

5.2. Introduction

Effective treatment of asthma remains a challenge especially in patients with severe disease who represent 5-10% of asthmatics. Despite optimal treatment these patients suffer significant mortality and morbidity from the disease and account for almost 50% of the economic burden of asthma, which, in the UK, amounts to more than £1 billion annually in direct heath care cost. Although airway smooth muscle (ASM) remodelling in asthma has been described almost 100 years ago, until recently no treatment has targeted at this process [4]. Since the realisation of significance of airway inflammation in asthma, glucocorticoids have become the cornerstone of asthma treatment especially since the 1970s when inhaled glucocorticoids (ICS) were introduced. Although ICS provide adequate control to most asthmatics this is insufficient in severe asthma. Furthermore, ICS have not been shown to alter airway remodelling beyond affecting inflammation-related changes.

In asthma, ASM structural abnormalities are evident even in mild disease. These include increased ASM mass due to hypertrophy and hyperplasia, and possibly augmented ASM contractility [61]. Unarguably ASM plays a significant role in the pathophysiology of asthma. This is simply evident by the central role bronchodilators play in the treatment of asthma. Short and long acting *beta*2 agonists relax ASM, improve symptoms and improve lung function. However, this effect is temporary as these medications do not alter the fundamental structural abnormalities of the airway namely airway inflammation and airway remodelling.

Bronchial thermoplasty (BT), where radiofrequency energy is directly directed to heat the large airways, is a novel complementary minimally invasive bronchoscopic treatment for patients with severe persistent asthma who are poorly controlled by ICS and bronchodilators. BT has been shown to improve asthma-related quality of life and also reduce both severe asthma exacerbations and hospital admissions [287]. The procedure is performed in three outpatient visits at least 3 weeks apart, sequentially treating separate parts of the lung. In the UK, This treatment has been

licensed by the National Institute for Health and Clinical Excellence and is available at some tertiary NHS hospitals for selected severe asthma patients as part of their clinical management. Although, it is thought that BT exerts its effect by reducing ASM mass, this fails to explain the improvement in exacerbations, a primary function of inflammation, demonstrated is BT studies. Moreover, the lack of improvements in lung function post BT remains unexplained [290]. Airway thermal injury and subsequent repair after BT represents a perfect model for improving our understanding of airway remodelling in asthma.

To the best of our knowledge, examining the dynamics of the airway structural changes measures by bronchial biopsy throughout the BT course in asthmatics has never been previously described. Additionally little is known about the effect of BT on airway morphometry and on air trapping indices measured by quantitative computed tomography (QCT). The aim of this study is to examine the immunopathological and imaging dynamics of airway injury and repair following bronchial thermoplasty, hypothesising that BT exerts its beneficial effects by means other than reducing ASM mass.

5.3. Methods

5.3.1. Study population

The study population included male and female asthmatic patients who were undergoing bronchial thermoplasty as part of their standard clinical care. Patients were identified and referred by the treating clinical team. The decision to treat these patients with BT was made by the clinical teams based on the patients' clinical needs. Potential participants received written patient information sheets at least 24 hours before signing the consent form. Inclusion criteria included 1) Males and females of any race who are over the age of 18 years at the time informed the consent is obtained, 2) Physician diagnosis of asthma, as per GINA guidelines and on GINA steps 3-5 asthma therapy; and 3) Patients assigned by the clinical team to receive bronchial thermoplasty as part of their asthma treatment plan. Patients with

current or recent (within 4 weeks of visit 1) lower respiratory tract infection or asthma exacerbation were excluded from the study.

The study protocol was approved by the National Research Ethics Committee (Leicestershire, Northamptonshire and Rutland). The study was registered with ISRCTN (http://www.isrctn.com/ISRCTN94263922).

5.3.2. Study design

This was a multicentre observational prospective study. BT was done as per each local centre clinical protocol. Patient who were assigned to receive BT by the treating clinicians as part of asthma treatment were identified and invited to participate in the stud. BT was performed as previously described [287]. The intervention is normally performed in three separate bronchoscopic sessions at least 3 weeks apart treating different parts of the lung sequentially. Each lower lobe is treated in the first 2 sessions and both upper lobes are treated during the third session.

The study had 5 visits. Each of the first three visits corresponded to a BT session, with all assessments performed prior to the BT session, while visit 4 is 6 weeks post the final BT session. Bronchial biopsies were obtained during the BT session (after finishing RF energy delivery) from areas not treated during the same session. This allowed the examination of the airway tissue prior to treatment and at two different time points post treatment. Visit 5 was done 6 months after completing BT.

5.3.3. Qualitative computed tomography

Inspiratory and expiratory scans were obtained at two time points, prior to BT and at 6 weeks (visit 4) post BT completion. As previously described, expiratory scans were used to obtain quantitative analysis of air trapping, while inspiratory scans allow the quantification of bronchial wall and lumen measurements. Scans were analysed using the semi-automated Apollo software (VIDA Diagnostics, Iowa).

Subject's individual levels of radiation exposure were monitored during the course of the study. The maximum allowed radiation dose was 10mSv over the course of this study.

5.3.4. Pulmonary function and sputum induction

Spirometry was performed according to the American Thoracic Society / European Respiratory Society (ATS / ERS) guidelines [296,297]. Post bronchodilator spirometry was done twenty minutes after the inhalation of 400µg salbutamol. Sputum induction and processing was performed as previously described [298]. The samples were analysed by a single blinded operator and the differential cell counts of epithelial cells, lymphocytes, eosinophils and neutrophils were recorded and expressed as percentage values of a sample containing at least 400 non-squamous cells.

5.3.5. Asthma control and asthma quality of life

Asthma control was measured using the previously described Juniper Asthma Control Questionnaire (ACQ). ACQ is simple validated tool for quantifying asthma control with a recall period of 1 week [301]. It is composed of 7 items; the first 5 are self-administered and assess night and daytime symptoms and activity limitation. Item 6 evaluates short acting bronchodilator use while question 7 is concerned with the degree of airflow limitation (FEV_1 %predicted).

Asthma quality of life was measured using the well validated Juniper Asthma Quality of Life Questionnaire (AQLQ). The questionnaire is composed of a total of 32 items which cover 4 functional domains: Activity Limitation, Emotional Function, and Environmental Exposure. The standardised version of the AQLQ was used. The score for each question is given in a 7 point scale (higher score indicating better quality of life). An overall AQLQ score was also obtained as the mean of all 32 items scores. As previously validated, a value of 0.5 was considered as the minimally important difference [291].

5.3.6. Bronchial biopsy

Bronchial biopsies were obtained at each of the three BT sessions. At each session biopsies were obtained following the completion of the BT process, if deemed safe by the bronchoscpist. Samples were obtained from areas not treated during the same BT session. As previously described, biopsies were fixed immediately for 24 hours in 10% Neutral buffered formalin and then embedded in paraffin blocks [303].

Four micrometre sections were cut from the biopsy blocks and stained with Haematoxylin & Eosin (H&E). Immunohistochemical staining was done with anti-*alpha*-smooth muscle actin (α-SMA) clone 1A4 (Dako, UK), and appropriate isotype controls. The endobronchial biopsies were assessed by a single observer blinded to the clinical characteristics (ZEN 2012 image analysis software for light microscopy, Carl Zeiss AG, Germany) and expressed as the mean of measurements undertaken from a minimum of two sections either from independent biopsies or as non-contiguous tissue sections at least 20μm apart from the same biopsy block. Epithelial integrity was assessed by measuring the length of intact and denuded epithelium. These were expressed as percentage of all the total reticular basement membrane (RBM) length present in the section. RBM and epithelial thickness were measured as described previously [113]. Tissue areas were measured on H&E and each area expressed as percentage of the total section area. Areas stained with actin were assessed using a thresholding technique based on the hue, saturation, and intensity (HSI) method for colour detection [304]. The HSI colour scale ranged from 0 to 360 for hue and 0 to 255 for both saturation and intensity. Images were acquired at ×100 magnification following correction for white balance. After validation, the following HIS setting was applied: hue (upper limit, 300; lower limit, 7), saturation (upper limit, 255; lower limit, 10), and intensity (upper limit, 30; lower limit, 8). The quantification of α-SMA expression was assessed as percentage of ASM area (Actin/asm%) and total section area (Actin/total%) occupied by α-SMA stained areas which are deemed to be above the predetermined threshold.

5.4. Results

5.4.1. Patients characteristics and demographics

12 patients were enrolled in the study from 4 different severe asthma tertiary centres in the UK. Patients' characteristics are summarised in Table 5.1. All the patients fulfilled the ATS/ERS criteria for severe refractory asthma with 7 patients (58%) on step 5 of the GINA treatment guidelines and the remaining were on step 4 [5]. No patient was on anti-IgE therapy and none had previous BT. Seven patients were (58%) female. Patients' age ranged 21-58 years. All the patients were non-smokers with seven patients (58%) never smokers and five ex-smokers (0.5, 1.2,6,8 and 10.0 pack years).

5.4.2. Asthma control, asthma quality of life and lung function

There were no significant statistical or clinically relevant differences in the mean ACQ7, ACQ6 or AQLQ between pre-BT, 6 Weeks post BT or 6 months post BT (Figure 5.1). Similarly BT did not result in any significant changes in pre- and post bronchodilator FEV_1, Pre- and post bronchodilator FEV_1/FVC or carbon monoxide diffusion capacity (TLCO) and transfer coefficient (KCO). See Figure 5.2 and Table 5.2.

5.4.3. Bronchial biopsy

Bronchial biopsies were obtained from 5 subjects. No adverse reaction resulted from obtaining tissue samples during BT sessions. Samples from the first BT session were obtained from any area not treated during the session (right lower lobe). Samples during second and third BT session were obtained from the right lower lobe which was treated during the first BT session.

5.4.3.1. Airway smooth muscle changes

It was observed that although α-SMA staining was normal in sections of biopsies obtained in the first BT session with all the ASM bundles areas well stained, however, this was not the case in samples obtained in the second and third BT sessions where only parts of the ASM areas retained the staining. (See Figure 5.3).

Tissue areas were thus measured in H&E sections. To validate measuring tissue areas using H&E stained sections, a validation cohort was used in a separate cohort of 10 biopsies (non-BT subjects), where ASM% was measured in both H&E and α-SMA stained sections (obtained from the same block and cut <20μm apart) . The intra-class correlation coefficient, using a two-way random effect model with absolute agreement, of ASM% measured in H&E and α-SMA stained sections was 0.99 ($p<0.001$).

Mean ASM%, as measured on H&E sections, did not change significantly throughout the BT treatment (BT1=25.2 (7.8), BT3=19.8 (19.3), p=0.63). However, acting staining as measured by actin thresholding reduced significantly post BT. There was a clear trend of reduction in actin area when expressed as actin area/ASM(%) and actin area/total area(%) with statistically significant mean reduction of both indices at BT3 compared to BT1 (BT1= 45.9 (15.8), BT3=11.8 (9.7), p=0.035 and BT1=10.3 (2.6), BT3=2.7 (3.2), p=0.027, respectively). See Figure 5.4.

5.4.3.2. Epithelial changes

RBM thickness did not change significantly post BT (BT1= 8.5 (2.3), BT3= 6.6 (2.1), p=0.082). Epithelial integrity improved post BT as there was a significant improvement in mean intact epithelium% at both BT2 and BT3 compared to BT1 (p=0.017 and 0.04) (BT1= 11.6 (11.3), BT2= 46.9 (21.9), BT3= 48.0 (22.6)). Similarly, denuded epithelium% was significantly reduced at BT2 and BT3 compared to pre-treatment (p=0.07 and 0.023 respectively) (BT1= 28.7 (22.2), BT2= 7.3 (4.6), BT3=12.2 (10.0)). Epithelial thickness was not significantly affected by BT. See Figure 5.4.

5.4.4. Quantitative computed tomography

There was no difference in any of the airway morphometry or lung densitometry markers between pre and 6 weeks post BT. Thus there was no significant change lumen area, wall area, wall area %, MLD E/I or VI-856 HU.

Table 5.1: Patients demographics and baseline characteristics.

Characteristic		**Subjects (n = 12)**
Age (y)		45.6 (15.1)
Male (%)		41.7
Asthma duration (y)		24.5 (21.1)
BMI (kg/m^2)		28.5 (6.4)
Exacerbations in the last 12 months		5.6 (3.9)
Age of asthma onset (y)		15.5 (14.3)
Ex-smokers (%)		41.7
Atopy (%)		67
GINA class	**GINA 5 (%)**	58.3
	GINA 4 (%)	41.7
	GINA 3 (%)	0
	GINA 1&2 (%)	0
Inhaled BDP equivalent (µg/24h)		1808 (1055)
Pre-Bronchodilator FEV_1 (L)		2.19 (0.84)
Pre-Bronchodilator FEV_1 (% predicted)		69.3 (21.2)
Pre-Bronchodilator FEV_1/FVC (%)		59.4 (14.4)
Post-Bronchodilator FEV_1 (L)		2.54 (0.76)
Post-Bronchodilator FEV_1 (% predicted)		81.1 (17.7)
Post-Bronchodilator FEV_1/FVC (%)		62.25 (12.8)
FEV_1 reversibility		20.3 (15.1)
KCO (%)		109.3 (11.1)
TLCO (%)		94.8 (10.1)
€VI-856HU		9.1 (12.1)

Table 5.2: Symptom scores and lung function parameters pre- and post BT.

Outcome	**Baseline values**	**6 Weeks post BT**	**6 Months post BT**	**P value**
ACQ-7 score	3.18 (1.27)	3.22 (1.34)	3.27 (1.28)	0.69
ACQ-6 score	3.14 (1.4)	3.32 (1.31)	3.00 (1.3)	0.86
AQLQ score Total	3.77 (1.44)	3.52 (1.38)	3.34 (1.42)	0.93
AQLQ score Symptoms	3.63 (1.36)	3.43 (1.38)	3.44 (1.42)	0.92
AQLQ score Activities	3.97 (1.62)	3.69 (1.36)	3.55 (1.5)	0.53
AQLQ score Emotions	3.52 (1.7)	3.38 (1.75)	3.13 (1.69)	0.86
AQLQ score Environmental	3.9 (1.63)	3.53 (1.64)	3.28 (1.91)	0.67
Pre-bronchodilator FEV_1 (L)	2.19 (0.84)	1.66 (0.57)	1.88 (0.71)	0.85
Pre-bronchodilator FEV_1 (% predicted)	69.2 (21.2)	56.8 (20.2)	61.0 (18.4)	0.43
Pre-bronchodilator FEV_1/FVC (%)	59.4 (14.4)	53.9 (12.6)	56.4 (11.4)	0.36
Post-bronchodilator FEV_1 (L)	2.54 (0.76)	2.35 (0.72)	2.26 (0.74)	0.7
Post-bronchodilator FEV_1 (% predicted)	81.1 (17.7)	75.2 (19.4)	72.06 (17.0)	0.72
Post-bronchodilator FEV_1/FVC (%)	62.25 (12.8)	61.1 (11.4)	60.2 (9.5)	0.52
FEV_1 reversibility (%)	20.3 (15.1)	30.3 (23.3)	27.2 (18.4)	0.85
TLCO (%)	94.8 (10.1)	89.4 (14.4)	90.6 (5.4)	0.93
KCO (%)	109.3 (11.1)	110.3 (16.5)	106.2 (20.2)	0.78

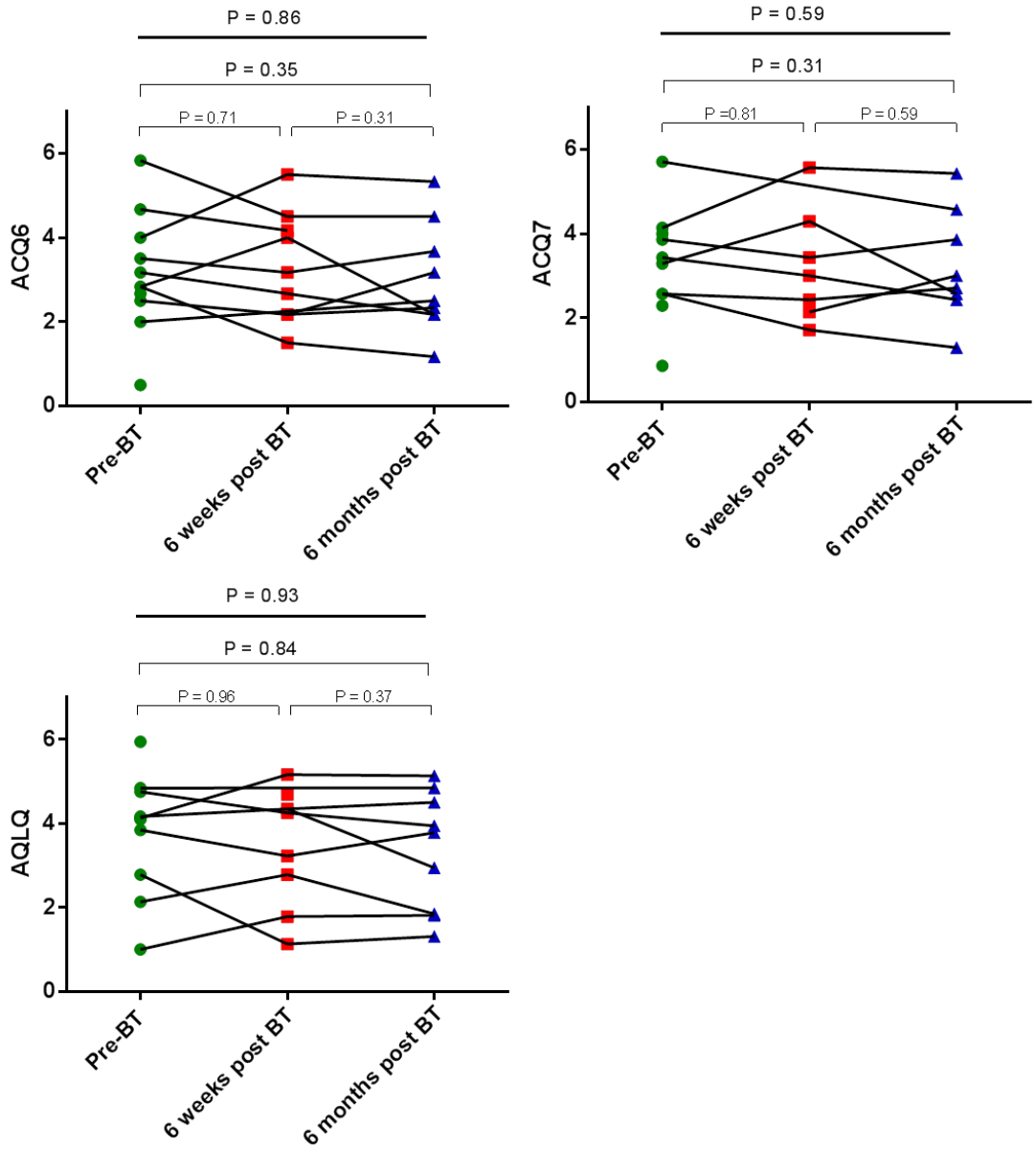

Figure 5.1: Changes in asthma control questionnaire scores (ACQ6 and ACQ7) and asthma quality of life questionnaire (AQLQ) pre- and post BT completion. No statistically significant changes were seen. Paired t-test was used when 2 groups were compared while one-way ANOVA was used when > 2 groups were compared.

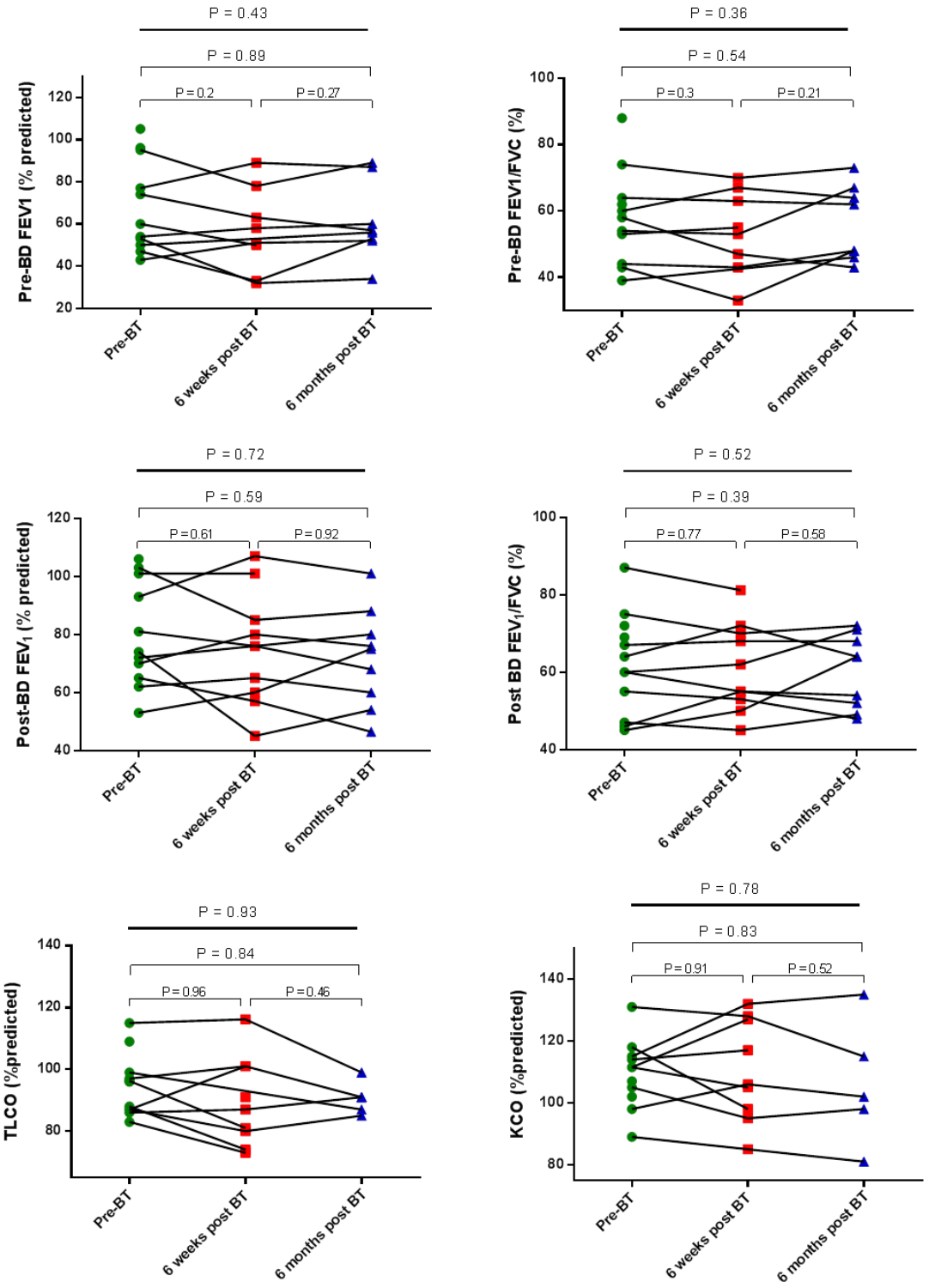

Figure 5.2: Lung function testing parameters pre- and post BT. BT did results in any significant changes in all the measured lung function indices.

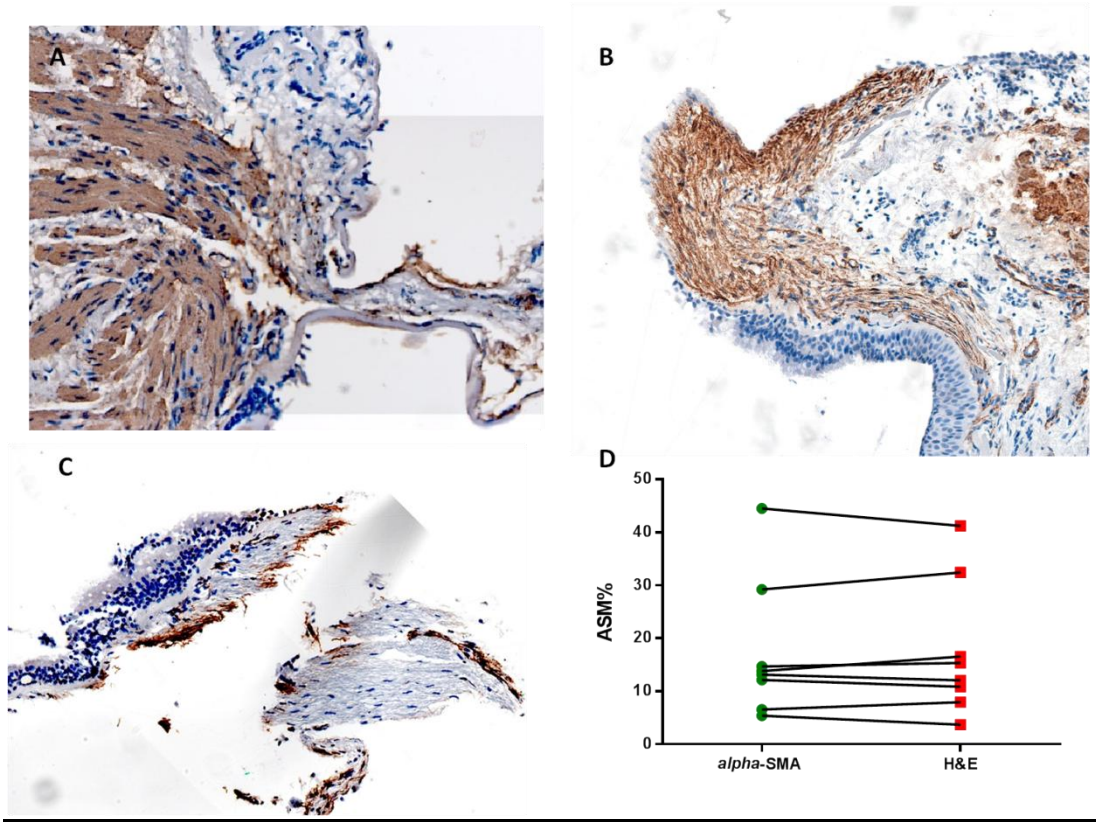

Figure 5.3: Panels A, B and C showing photomicrographs of biopsy sections stained with *alpha*-smooth muscle actin (α-ASM). (A) was obtained at BT session 1 (pre-thermoplasty) showing normal α-ASM staining pattern, while (B) and (C) were from BT sessions 2 and 3 respectively and are both demonstrating reduced α-ASM staining. Panel D shows the correlation of airway smooth muscle % measured on H&E and α-SMA stained sections. The intra-class correlation coefficient = 0.99 ($p<0.001$).

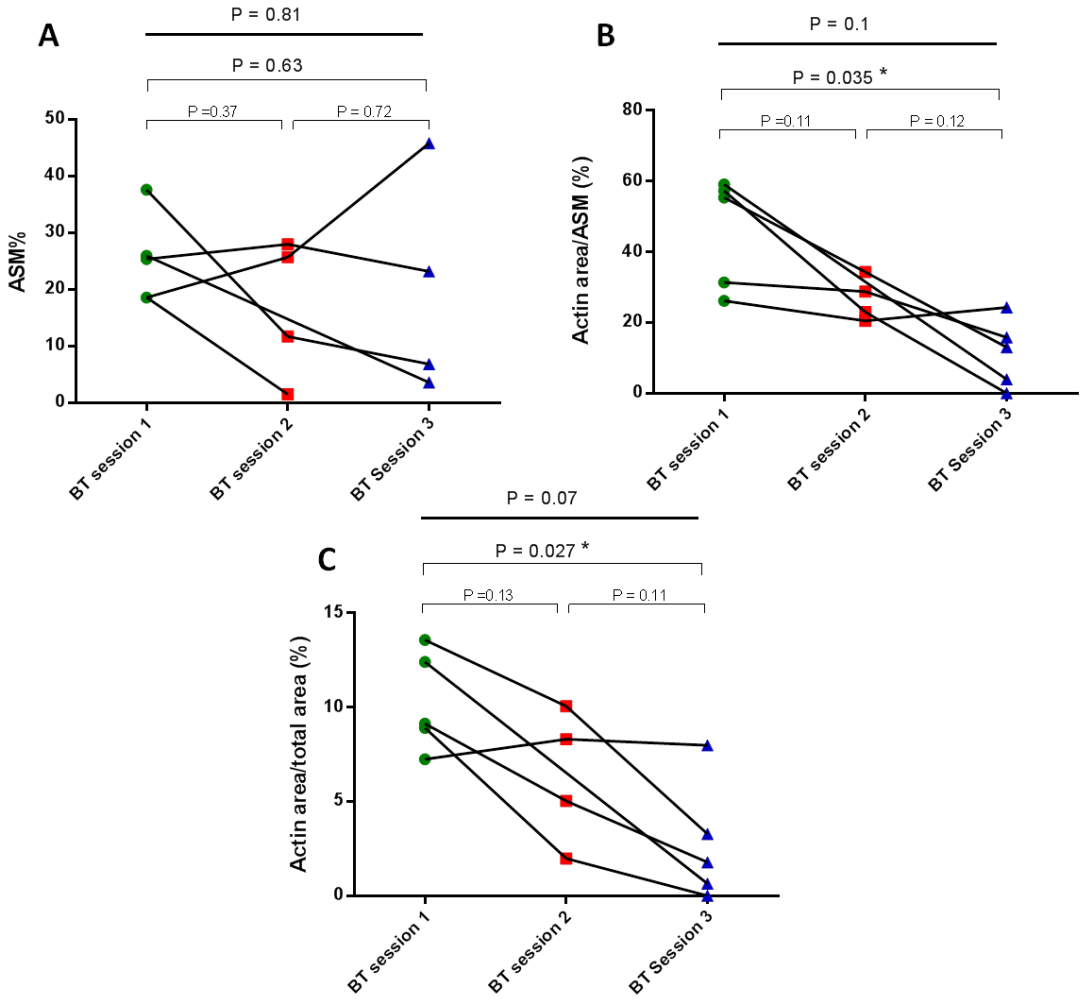

Figure 5.4: Effect of thermoplasty on (A) ASM% measured on H&E, (B) actin area/ASM expressed as a percentage of the area of actin staining measured by thresholding from ASM area measured on H&E sections and (C) Actin area measured by thresholding as a percentage of the total section area.

Table 5.3: Airway remodelling outcomes from BT1 (Pre-BT) and BT3 (Post-BT) biopsy samples.

Remodelling Outcome	**BT1**	**BT3**	**P value**
ASM%	25.2 (7.8)	19.8 (19.3)	0.63
Actin/ Total area (%)	10.3 (2.6)	2.7 (3.2)	**0.027**
Actin/ASM area (%)	45.9 (15.8)	11.8 (9.7)	**0.035**
RBM thickness	8.5 (2.3)	6.6 (2.1)	0.82
Denuded epithelium	28.7 (22.2)	12.1 (10.0)	0.23
Intact epithelium	11.6 (11.3)	48.0 (22.6)	**0.04**
Epithelial thickness	37.7 (9.8)	40.3 (2.8)	0.56

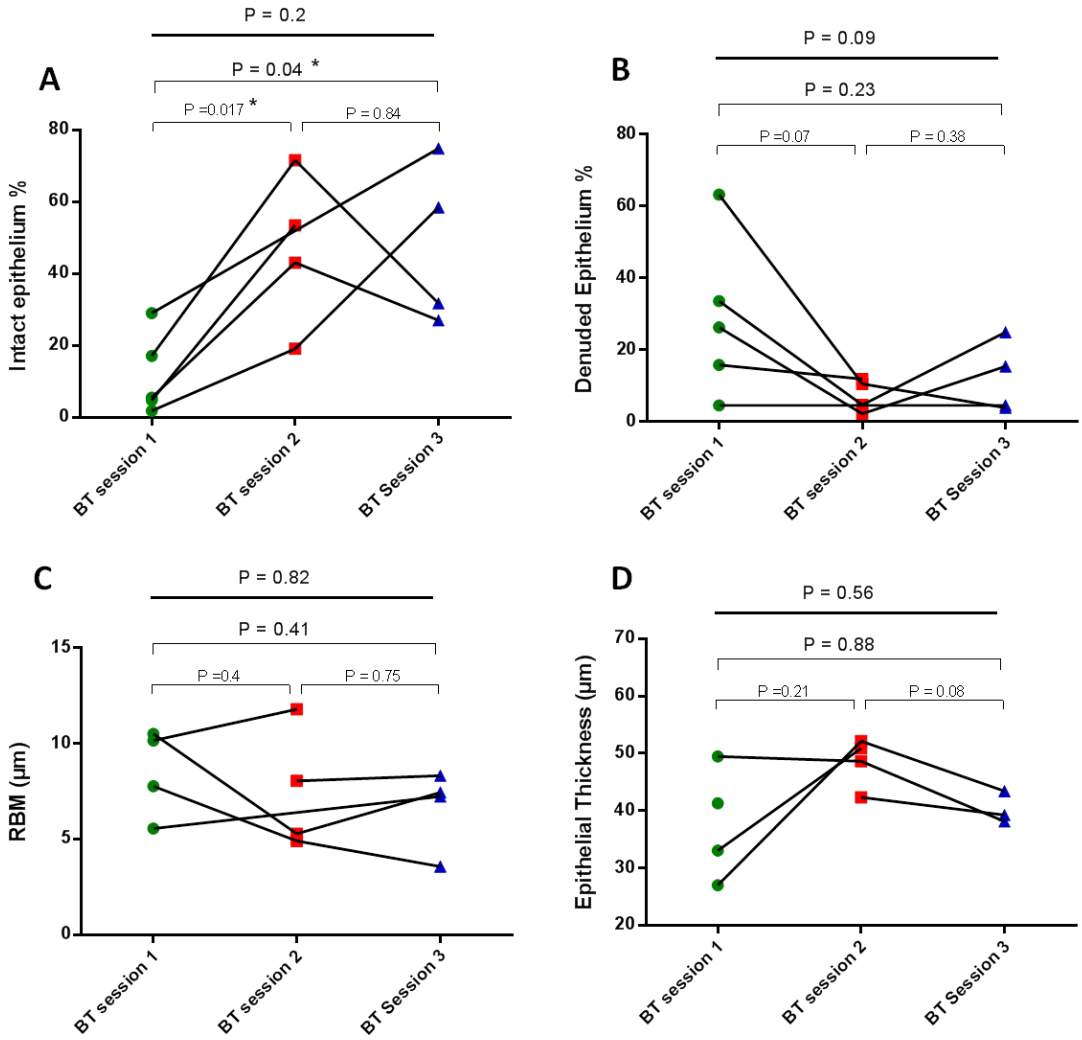

Figure 5.5: Epithelial changes pre- and post BT; (A) intact epithelium%, (B) denuded epithelium%, (C) RBM thickness and (D) epithelial thickness.

5.5. Discussion

In this study, we reported changes in lung function, asthma symptoms, QCT indices of morphometry and air trapping, and biopsy-derived airway structure in a group of severe asthmatics treated with bronchial thermoplasty. We found that there was no significant difference in ACQ, AQLQ, TLCO, KCO, FEV_1 %predicted or FEV_1/FVC when measured pre-BT, 6 Weeks and 6 months post BT.

Airway remodelling measured on bronchial biopsies in 5 subjects collected during the three BT sessions, (in BT1 biopsies were taken from untreated airways, in BT2 samples were taken from areas treated at BT1 and in BT3 samples were taken again from areas treated at BT1), showed that ASM% did not change when measured on H&E stained sections. However, we observed altered actin expression after BT. Actin stained area was altered post BT with significant reduction in the following measurements: actin area/ASM(%) and actin area/total area(%). This could be the result of contractile proteins denaturation as proposed by a previous *in vetro* study [336]. Whether this will eventually lead to apoptosis of ASM cells is unknown, however, this study showed that 1-3 months after BT (the time range between BT1 and BT3) ASM mass did not change.

Our study has shown significant alterations to the airway epithelium post BT. There was significant improvement in intact epithelium and reduction in denuded epithelium. This follows the extreme epithelial injury resulting from BT which can be seen with the naked eye during BT with immediate blanching of the treated area. This suggests the possibility that epithelium repair post thermal injury leads to resetting of the epithelium to a healthier state compared to pre-BT. The airway epithelium in asthma is known to be abnormal and fragile with areas where the basement membrane is denuded and exposed [337]. The epithelium normally acts as a physical barrier against irritants and stimulates that can lead to worsening of the airway inflammation and possibly leading to asthma exacerbations. These include allergens that, without an intact epithelium, would have direct access to the

subepithelial dendritic cells, the chief antigen presenting cells in the lung [338]. This taken together, it would be reasonable to suggest that the improvements in asthma exacerbations seen in patients treated with BT in previous studies could be due improvement in quality of the epithelium rather than due to changes in the ASM, although this remains a hypothesis and further research is needed to investigate and prove this.

The lack of effect on lung function and asthma control is not surprising. A recent large systematic review, which included the only three randomised controlled trial on BT (AIR, AIR2 and RISA studies), did not show any significant improvement in pulmonary function parameters or ACQ scores [292]. BT has always been presented as an anti-remodelling treatment that targets ASM, however, the lack of improvement in lung function in all the major studies of BT casts doubts over this proposed mechanism of action. It is intriguing that we could not show any effect from BT on AQLQ. However, most studies have shown only a modest improvement in AQLQ following BT, and our study was not powered to show such a signal.

This work clearly has some potential limitations. Despite being a multi-centre study, it only included 12 subjects. This is due to BT being a relatively new licensed therapy in the UK with only a few centres offering the treatment to a small number of selected severe asthmatics. Due to various factors, less than half of the subjects in our study underwent airway tissue sampling. Some of the clinicians had limited experience in performing BT and, as a result, expressed reluctance to obtaining tissue samples following the delivery of the radiofrequency treatment during the BT session. Furthermore, BT can cause immediate adverse effects resulting in bronchospasm and worsening of the patient's clinical condition. This has also contributed to the low rates of tissue sampling. Our work has looked at the acute effects of BT on remodelling, however, further studies are needed to investigate longer term effects by obtaining airway samples 6-12 months after BT which would significantly help clarify the different roles ASM and epithelium changes play in the

resulting benefits seen from BT. Notwithstanding the obvious shortcomings, our work has shown novel aspects of BT effects on various components of the airway structure that might help shed some light on the role of remodelling in the pathogenesis of asthma.

In conclusion, we have shown in this small study, that BT improves epithelial integrity. Whether this improved airway epithelium in response to thermal injury is sustained or may have any clinical impact on asthma control or exacerbation rate requires further investigation. Furthermore, our data showed reduced actin expression on biopsies suggesting possible denaturation of contractile proteins as a result of BT. The long term sequelae of this on the different asthma domains is unknown and needs to be studied.

6. CHAPTER SIX: DISCUSSION, CONCLUSIONS AND FUTURE

6.1. Introduction

Asthma remains a significant disease worldwide with an annual estimated 250,000 deaths directly attributed to asthma globally. The disease affects people of all ages in all regions of the world. The burden asthma includes significant mortality, morbidity and huge economic costs all of which affect mainly patients in the severe spectrum of asthma. The main reason for this is the lack of effective new treatments and therapies that are better or comparable in efficacy to inhaled corticosteroids (ICS); a treatment category developed more than 40 years ago and yet remains the cornerstone of asthma drug therapy. All novel treatments developed after ICS, like leukotriene antagonists and anti-IgE antibodies, remain complementary and are not as effective as ICS. In the past, better understanding of the role of inflammation has led to better management of asthma. In fact ICS, acting as an anti-inflammatory, are sufficient in most non-severe asthmatics who account for about 90% of patients. However, a huge demand for novel therapies still exists to help treat the remaining 10% who have severe refractory asthma.

Airway remodelling was always thought to be secondary to inflammation and although it has been first observed almost a century ago, until recently no treatment has been developed to target the process. Recent evidence showing that elements of airway remodelling, at least in part, are independent of inflammation, coupled with little prospect of improving the way inflammation is targeted in asthma, have led to renewed interest in studying airway remodelling.

The central focus of this thesis has been the role of airway remodelling in asthma. This process is poorly defined, poorly understood and arguably remains the main obstacle in understanding asthma natural history and pathogenesis and, hence, developing desperately needed new asthma therapies. Several challenges arise when trying to study airway remodelling. The term defines heterogeneous

microscopic changes that are, in part, poorly connected and occupy different roles in both the pathophysiology and natural history. Moreover, these various elements have different relationship to airway inflammation with some closely connected to it (increased RBM thickness) while others are not (e.g. ASM hyperplasia).

This thesis attempted to answer apparently simple questions that collectively would help define the role on airway remodelling in asthma. In the first study I attempted to unravel the link between airway remodelling and the physiological changes seen in asthma. This was coupled with trying to examine the associations of large and small airway changes seen on qualitative computed tomography (QCT) and airway remodelling seen on endobronchial biopsy. In the second study I examined the effect of a newly developed PD_2 antagonist, fevipiprant, on eosinophilic inflammation, asthma control, asthma quality of life, lung function and, more importantly, airway remodelling. In the last study, I reported the effects of the only licensed non-drug device in the treatment in asthma, bronchial thermoplasty (BT), on airway remodelling, challenging the largely agreed opinion suggested by earlier studies that BT exerts its beneficial effect through its action on ASM. In this concluding chapter, I will be summarising the results of all the included studies, highlighting important limitations and challenges, and finally suggesting future research directions.

6.2. The relationship between airway remodelling and physiology in asthma

Most agree that airflow obstruction is the most important biomarker of asthma. It is the primary defining characteristic of the disease with fixed airflow obstruction being a strong predictor of asthma control, asthma severity, exacerbation rate and decline. Consequently, to evaluate any pathological process or biomarker of asthma, as airway remodelling in this thesis, it is crucial to investigate its association with airflow obstruction.

To achieve this we measured airway remodelling and inflammation markers on endobronchial biopsies and compared these with indices of airflow obstruction, namely FEV_1 and FEV_1/FVC, in 70 asthmatics of variable severity. Simple univariate correlation showed significant negative correlation of ASM and vascular remodelling, measured by the mean Chalkley count, a surrogate marker of neoangiogenesis, with both FEV_1 and FEV_1/FVC. Moreover, in comparing subjects with and without fixed airflow obstruction, defined as post bronchodilator FEV_1<80%, there was significant difference in ASM% and vascularity. There was no significant difference between the two groups in any of the other inflammatory or remodelling markers. Using step-wise regression analysis has shown that only ASM% was an independent predictor of post bronchodilator FEV_1 while both ASM% and vascularity were significant predictors of post-bronchodilator FEV_1/FVC.

Evidence from previous studies have shown mixed results when studying the association of ASM remodelling and lung function. Furthermore, few small studies have assessed the relationship between vascular remodelling and lung function. This study is one of the largest to study biopsy measured remodelling and hence the results carry more significance. The lack of relationship between RBM thickness and lung function is interesting. Similarly to ASM, RBM thickness has been shown to be associated with lung function changes in some but not all studies [72,304]. RBM thickness in itself would not contribute to airway narrowing (usually <30μm) but is considered as a marker of the mesenchymal changes (fibrosis) seen in the airway.

6.3. Airway remodelling and QCT, from microscopic to macroscopic

Accurately assessing and measuring airway remodelling remains the central challenge in studying the role of the process in the pathogenesis, disease mechanism and clinical expression of asthma. Historically post-mortem studies were the primary means of clinical research into airway remodelling, however, this method has its obvious drawbacks. Histological studies using endobronchial biopsies (EBBs) collected mainly from the carina of proximal large airways, remains the gold

standard method of studying airway remodelling. Although using EBBs carries the advantage of being able to study inflammation alongside remodelling on the same samples, thus helping clarify the relationship between the two processes, and being able to directly measuring remodelling changes and markers; the method has a number of significant limitations. EBBs allow only limited insight into the composition of the large airways as they are small biopsies obtained from the superficial surface of the airway and not cross-sectional or full thickness. Moreover, EBBs are even more limited in studying changes in the small airways, an important physiological aspect of asthma. Although some of the remodelling changes are present in both small and large airways, significant differences remain.

All the above highlights the need for new non-invasive image-based methods to study airway remodelling in asthma. The advancement in multi-detector CT coupled with new post-processing software technology have allows the quantitative assessment of large airway morphometry and small and indirectly measure small airway disease thorough the quantification of air trapping. QCT indices correlate well with lung function changes and with asthma severity [124]. Only a very small number of studied the association between QCT metrics and airway remodelling measured on biopsy.

To our knowledge the study included in this thesis is the largest study to date comparing immunohistology, including both airway remodelling and inflammation, with QCT parameters. It is also the first airway remodelling study to include QCT markers of air trapping. In our study we have shown that vascularity as measured by the Chalkley count, a well validated stereological method to assess neoangiogenesis, and to a lesser extent airway smooth muscle content, were associated with indices of air trapping. Using step-wise multiple regression analysis we have shown that vascularity was the only independent predictor of MLD E/I, arguably the most significant airway trapping QCT-derived metric. This relationship, which to our knowledge has never previously been described, was validated on an independent

group of asthmatic where the mean Chalkley count strongly correlated with MELD E/I (R=.44, p=0.031). Vascularity was not related to any proximal airway QCT morphometry changes. Epithelial thickness and airway smooth muscle content both correlated to morphometry changes with both shown to be predictors of mean segmental bronchial luminal area while epithelial thickness was the only independent predictor of mean segmental bronchial wall area %. Interestingly, neither airway inflammation nor RBM thickness were shown to have any association with QCT-derived measurements. Although only a small number of biopsies had adequate intact epithelium to allow goblet cells enumeration, there was very strong correlation of goblet cell hyperplasia, a known remodelling feature in asthma, and air trapping markers. Due to the limited numbers, this result should be interpreted with caution.

The above methods used in addressing the questions around the relationship between airway remodelling and 1/ lung function, and 2/ QCT measurements (sections 6.2 and 6.3 respectively), have a number of potential limitations. Although this study is, to date, one of the largest to address the physiological relevance of airway remodelling in asthma and the largest to investigate the link between microscopic changes and QCT in asthma, it is still considered a relatively small study. It is also retrospective and cross-sectional, thus not ideal to address a complicated question like structure-function relationship in asthma. The lack to symptoms parameters like asthma control and asthma quality of life scores from the results is another significant limitation. The use of EBBs carries inherent limitations. Despite being the best method of studying remodelling, these biopsies are small and only sample the superficial layer of the airway, thus cannot determine the changes in airway structure in relationship to the whole depth of the airway wall. Furthermore, we have not standardise the location of the biopsies obtained to correspond with the airway morphometry measured by QCT and whether this is important to determine the heterogeneity within an individual will be important to explore in future studies.

While most of the remodelling measures used in this study were extensively used in previous studies, e.g. RBM thickness, other markers like epithelial integrity and goblet cells measures were developed and suggested during conducting the studies of this thesis. While this is potentially a significant contribution to the field of remodelling research, it could be considered a limitation and these markers will need to be further validated and standardised in future studies.

In conclusion, we have found important associations between endobronchial biopsy and QCT measures of airway remodelling with lung function. We found that ASM mass and airway vascularity are related to airflow obstruction, with ASM mass likely contributing more to large than small airway remodelling, whereas increased vascularity appears to be related to air trapping possibly due to small airway remodelling. Although epithelial remodelling was found to be associated with QCT changes, correlation of epithelial thickness with morphometry and goblet cell counts with air trapping, no link was found between these changes and lung function. The association of these changes could be with other asthma domains not measured in this study like asthma exacerbations and asthma symptoms. These need to be explored with future longitudinal studies.

6.4. DP_2 antagonism in asthma

Prostaglandin D_2 (PGD_2), a lipid mediator derived from arachidonic acid through the cyclooxygenase pathway, plays a central role in the pathogenesis of asthma. It is mainly produced by activated mast cells. In addition to its direct effect on the airway, resulting in ASM contraction and airway tissue oedema, PGD_2 also contributes to the TH_2 cellular immune response seen in eosinophilic asthma through its action on DP_2 ($CHTH_2$) receptors on eosinophils, inducing their chemotaxis, survival and degranulation; and TH_2 lymphocytes, facilitating thier migration and inhibiting apoptosis. Given the significant role of PGD_2/DP_2 axis in asthma, it would be

reasonable to assume that inhibiting DP_2 would be beneficial as a treatment for the disease.

We conducted a double blind randomised controlled trial of fevipiprant, a highly selective DP_2 antagonist, in patients with moderate to severe eosinophilic asthma. After a run-in period patients were randomised on receiving 12 weeks of fevipiprant or placebo. This was followed by a single blinded 6 weeks wash out period. Given this was an early phase trial (phase II) of what practically is considered an anti-eosinophilic drug, the primary outcome was the reduction in eosinophils numbers in induced sputum in patients given fevipiprant compared to placebo. Secondary objectives were to determine the effects of fevipiprant on asthma control, asthma quality of life, lung function and importantly airway remodelling. This was a phase II trial so safety and tolerability were also an important outcome measure.

This study demonstrated that, in patients with persistent moderate-to-severe asthma and sputum eosinophilia, fevipiprant significantly reduced eosinophilic inflammation in the sputum and bronchial submucosa compared to placebo. Fevipiprant also significantly improved AQLQ(S) scores, post-bronchodilator FEV_1 and ACQ-7 scores , although the last was only in the sub-group of patients who had poor asthma control at baseline (ACQ-7 ≥ 1·5 points).

Airway tissue sampling was performed pre- and post-treatment in 26 subjects, 14 in the treatment arm and 12 in the placebo arm. Importantly fevipiprant improved epithelial integrity compared to placebo with significantly higher intact epithelium and lower denuded epithelium. Whether this is secondary to attenuation of TH_2 inflammation, as eosinophils are known to contribute to epithelial damage in asthma, or due to a direct effect of the drug on the epithelium is unknown, however, the lack of correlation between changes in eosinophilic inflammatory markers and epithelial integrity in response to fevipiprant favours a direct mechanistic effect upon the epithelium. Fevipiprant was also associated with improvement in RBM thickness of magnitude seen in studies using corticosteroids; however, this did reach

statistical significance. Intriguingly, there was significant reduction in ASM mass in the fevipiprant group compared to placebo. The mechanism underlying this effect is unknown and further studies are needed.

A major limitation of our study is the small sample size. This was a single centre study with 61 subjects enrolled. Biopsies were obtained only in 26 subjects. As the primary focus was tolerability and safety, the study was of a relatively short duration which did not allow capturing of exacerbation outcomes, an important effect of most anti-eosinophilic therapies (e.g. anti-IL5).

We have shown that, in patients with moderate-to-severe uncontrolled eosinophilic asthma, fevipiprant, a DP_2 receptor antagonist, is safe, tolerable and effective in reducing eosinophilic inflammation. Fevipiprant also improves lung function, asthma-related quality of life, asthma control and airway remodelling. The anti-remodelling effect of the drug is explained in part through its anti-eosinophilic but different mechanisms are possible. Longer-term multi-centre studies are required to confirm these findings and to investigate the effect of fevipiprant on asthma exacerbations and airway remodelling.

6.5. Bronchial thermoplasty and airway remodelling

Bronchial thermoplasty (BT) is a novel FDA-approved non-drug therapy for the treatment of severe asthma. The treatment involves the delivery of radiofrequency energy directly to proximal airways (>3mm in size) to heat the airway tissue to 65 degrees Celsius. This is claimed to reduce ASM mass leading to improvement in asthma symptoms and reduction in exacerbations and hospital admissions. BT is a bronchoscopic treatment that is delivered in three separate sessions treating different parts of the lung sequentially.

The evidence for safety and effectiveness of BT is from three clinical studies AIR, RISA and AIR2 trials [285,287,289]. The later was the largest trial of BT and the only sham controlled. AIR2 trail showed improvement in AQLQ, hospitalisation and

exacerbation rate. A systematic review and meta-analysis which included the three trials concluded that BT results only in modest clinical benefit [292]. BT has been shown to reduce ASM in early studies on animals and non-asthmatics. More recently, studies have shown some evidence of reduced ASM in asthmatics [293].

BT as a model of airway injury can help our understanding of the relevance of airway remodelling in asthma. Results of BT clinical studies have left many questions unanswered. For a treatment that targets ASM, it is peculiars that no improvement in lung function was seen in any of the studies. The aim of our study was to examine the immunopathological and imaging dynamics of airway injury and repair following bronchial thermoplasty.

Our study was observational and multicentre and only included patients that were assigned to have BT as part of their asthma management plan. Biopsies were obtained during the BT sessions where they were taken from non-treated areas at the first session and then from the area treated initially at the second and third sessions. This allowed the observation of the dynamic changes in the airway throughout the course of treatment. Patient also had pulmonary function testing, ACQ and AQLQ, and QCT pre- and post BT.

Biopsy analysis has shown that ASM mass was not affected when examined on H&E stained sections, however, actin staining was significantly reduced following BT suggesting possible denaturation of contractile proteins. Whether this eventually leads to ASM reduction was not shown by this study as no biopsies were collected in the period after BT completion. Importantly we have shown significant improvement in epithelial integrity with increase in intact epithelium and reduction in denuded epithelium. Most asthma exacerbations are induced by external stimulants like allergen or viral infection. Improvement in epithelial integrity could therefore explain the reduction in exacerbation seen in previous studies as it becomes more effective as a physical barrier.

Our study has significant limitations. The small number of subjects is the major shortcoming as only 12 patients were included and only 5 subjects underwent airway tissue sampling. The treatment is relatively novel in the UK with only a few centres offering it to a small number of highly selected patients. Attempting to obtain biopsies at the same session as BT, where the patient condition can deteriorate as a result of the BT treatment itself, also contributed to the small number of biopsies as in a significant number of patients it was deemed unsafe to proceed to biopsy. Although obtaining biopsies throughout the treatment is novel and has shown important results, our study did not address the issue of defining the longer term effects of BT on airway remodelling which needed airway sampling months after BT.

Our BT study attempted to use the thermal airway injury resulting from BT and the subsequent remodelling response as a model to simulate and further understand airway remodelling and repair in asthma; however, this has a few obvious drawbacks. The airway in asthma is exposed to injury from various other factors such as inflammation, infection and allergens, none of which could be simulated by simple heat. The asthmatic airways would never naturally be exposed to such temperatures and hence its response to natural stimulates is probably very different. Nonetheless, BT is the only treatment that primarily alters the structure of the airway with subsequent benefits; albeit modest, thus studying its exact effect would help us understand the relevance of remodelling.

Notwithstanding all the limitations, our study was the first to examine the dynamics of airway repair and changes in remodelling during BT. The epithelial improvement seen in our study warrants further research to examine whether this is sustained post BT and whether this has any effect on various asthma markers especially exacerbation rates.

6.6. Conclusion and future work

This thesis has attempted to add some answers to the ever elusive question of the significance and relevance of airway remodelling in asthma. Since the 1980s/90s, when the concept of Th_2 immunity/inflammation was introduced as the main mechanism underlying asthma pathogenesis, the focus in asthma research did largely shift to trying to better understand the biology and heterogeneity of airway inflammation with the introduction of various novel targeted anti-inflammatory agents that target key elements of Th_2 inflammation (e.g. anti-IL5 and anti-IgE). Although these agents have been shown to have some success in treating patients with severe asthma, this had been limited with the treatment being suitable for a small portion of asthma patients with modest results. This has been behind the current renewed interest in studying airway remodelling; as it is becoming clear that the more to asthma pathogenesis than inflammation. The relative success of bronchial thermoplasty, the first anti-remodelling therapy in asthma, shown in some clinical studies has also contributed to this revival of airway remodelling research. Furthermore, recent studies showing that at least some aspects of remodelling are independent from inflammation have raised questions on the exact role of the process in the natural history of asthma [14,17].

One of the main questions that this thesis has tried to address has been the physiological relevance of airway remodelling. We have described significant associations of vascularity and ASM remodelling with markers of airflow obstruction. This, to an extent, addresses the hypothesis that airway remodelling is significant determinant of abnormal airway physiology. Using multiple regression analysis we have shown that ASM and vascular remodelling are important determinants of the obstructive physiology seen in asthma. One of the main limitations of this study had been its cross-sectional nature. This has not allowed us to offer biological mechanisms explaining those biological correlations to clarify the position of airway remodelling in the cause-and-effect paradigm of asthma pathogenesis.

As the lack of tools to measure airway remodelling has always been one of the major hindrances to studying the process, we have attempted to assess the link between QCT-derived large and small airway markers with biopsy-measured structural changes. We found that ASM remodelling contributed to both morphometry and air trapping markers while vascularity was associated to the latter only. With the possibility of future advancement in CT scanning and post-processing technology we may be able to use more accurate machines that produce less radiation and thus allow us to conduct longitudinal studies to observe the dynamics of macroscopic airway changes especially in the context of clinical trials.

To help further understand the link between eosinophilic inflammation and remodelling we conducted an RCT of fevipiprant, a novel DP_2 antagonist. This was to address the hypothesis that airway remodelling could be targeted and modified by DP_2 antagonism. We have successfully demonstrated that fevipiprant reduces eosinophilic airway inflammation, improves lung function and symptoms and is well tolerated and safe in patients with persistent moderate-to-severe eosinophilic asthma. Furthermore, in addition to the expected improvements in inflammatory-related remodelling changes (e.g. RBM thickness); we have reported important changes in epithelial integrity and ASM mass. This has dictated the future need to further *in vivo* and *in vitro* studies in the role of PGD_2/DP_2 axis in epithelial repair and ASM remodelling. Some of these studies are already underway [339]. The two possible mechanisms of action by which DP_2 antagonism reduces ASM mass are either through a direct effect on ASM cells or indirectly through its inhibitory effect on eosinophils and TH_2 lymphocytes. The former seems more likely as no studies on the effects of any anti-eosinophilic therapy on airway remodelling have reported such sequelae on ASM mass (Table 1.3). On the other hand, mast cells, the primary source of PGD_2, are known to infiltrates ASM bundles in asthma; however, studies have failed to show any effect on ASM proliferation, migration or survival [28]. More importantly, PD_2 has yet to be shown to be expressed on ASM cells. On a more practical and clinically relevant point, fevipiprant is currently being tested in a

multicentre phase III trial which will better help define its efficacy and role as an asthma therapy.

In the last study, this thesis has explored the dynamic changes in airway remodelling in BT throughout the treatment course. Although this has been a small study it had findings that might help redefine the role of the epithelium in asthma. We have demonstrated that BT leads to improvement in epithelial integrity which could explain the reduction in exacerbations rates seen in BT trial although that needs to be confirmed with further studies. The results of this study partially refute and agree with our initial hypothesis. While we have shown that BT affects non-ASM structures, it also had an impact on ASM-related remodelling changes.

One of the major challenges for future asthma research is to attempt to develop better non-invasive tools to measure airway remodelling. Although bronchial biopsies are the gold standard, they remain significantly invasive, limited, controversial with non-standardised measures that are difficult to compare between different studies. These novel tools can be used on asthmatics of all stages and severity including children. This will help to redefine the role of the different heterogeneous components of airway remodelling in the pathogenesis and natural history of the disease. Unlike inflammation, biomarkers for airway remodelling in asthma are lacking. Future development of specific biomarkers for the various specific components of remodelling would help define the role they play in various asthma domains. Furthermore, biomarkers are also needed to help predict patients probably at a young age who are at risk of developing permanent remodelling changes that are commonly present in severe persistent asthma.

Asthma is increasingly recognised as a heterogeneous disease with different phenotypes, a phenotype being defines as "*set of observable characteristics of an individual resulting from the interaction of its genotype with the environment*". Although there is no universal agreement in the exact definitions and nature of these phenotypes, there are a few characteristics that seem to help differentiate and

define them. These include age of onset, atopy, Th_2 inflammation, susceptibility to infection, symptoms, lung function and weight. To help understand the relevance of these phenotypes and establish phenotype-specific therapy, it is important to explore the exact association with microscopic structural changes i.e. airway remodelling. Nonetheless, such evidence is lacking. This was not part of the studies included in this thesis and future research is needed to address the heterogeneity of airway remodelling in specific asthma phenotypes. This would help define new asthma endotypes with distinct biological signatures. This might help classify what we currently know as asthma into separate distinct diseases each investigated, assessed and treated differently leading to better outcomes.

Another future approach to help study and understand the structural changes in obstructive airways diseases, including asthma and COPD, is using computational modelling. Such multiscale micro and macro models can help address the whole biological complex cascade that results in the disease; genes to cells to tissue to organ and finally disease. By generating data at each biological scale and using computational and physical models to help integrate and interpret these data would help answer specific questions. The information needed to be included in these asthma models include genomics, transcriptomics, proteomics, lung function measurements of large and small airways, cross sectional imaging including QCT and functional MRI and biopsy measurements; which are generated and used by multidisciplinary teams from various fields. These can help create validated models that not only can help our understanding of the pathogenesis of asthma, but may even predict prognosis and disease progression.

This thesis has made significant original contributions to knowledge. It has made substantial contribution to the development of fevipiprant as a new treatment for asthma, validated methods to assess remodelling, demonstrated how remodelling relates to asthma outcomes and shown how fevipiprant and bronchial thermoplasty affect remodelling. The impact of this work on asthma is not aspirational, it is real

and potentially could translate into clinical practice in the very near future. Unsurprisingly, this thesis has also generated new questions that need to be addressed with further studies, some of which are already underway.

BIBLIOGRAPHY

1. Marketos SG, Ballas CN. Bronchial asthma in the medical literature of Greek antiquity *J Asthma* 1982; 19: 263-269.

2. Holgate ST. A brief history of asthma and its mechanisms to modern concepts of disease pathogenesis. *Allergy Asthma Immunol Res* 2010; 2: 165-171.

3. Sakula A. Henry Hyde Salter (1823-71): a biographical sketch *Thorax* 1985; 40: 887-888.

4. Huber H, Koesser K. The pathology of bronchial asthma. *Arch Intern Med* 1922; 30: 689-760.

5. 2015 GINA Report, Global Strategy for Asthma Management and Prevention - Global Initiative for Asthma - GINA http://ginasthma.org/gina-report-global-strategy-for-asthma-management-and-prevention/. Date last accessed: 4/10/2016 2016.

6. British guideline on the management of asthma. https://www.britthoracic.org.uk /document-library/clinical-information/asthma/btssign-asthma-guideline-2014/. Date last accessed: 4/10/2016 2016.

7. Masoli M, Fabian D, Holt S, Beasley R, Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report *Allergy* 2004; 59: 469-478.

8. Caughey GH. Chairman's summary. Mechanisms of airway remodeling. *Am J Respir Crit Care Med* 2001; 164: S26-7.

9. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics *J Clin Invest* 2003; 112: 1029-1036.

10. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, Gerard C. A critical role for eosinophils in allergic airways remodeling *Science* 2004; 305: 1776-1779.

11. Lowe LA, Simpson A, Woodcock A, Morris J, Murray CS, Custovic A, NAC Manchester Asthma and Allergy Study Group. Wheeze phenotypes and lung function in preschool children. *Am J Respir Crit Care Med* 2005; 171: 231-237.

12. Saglani S, Malmstrom K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, Turpeinen M, Rogers AV, Payne DN, Bush A, Haahtela T, Makela MJ, Jeffery PK. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. *Am J Respir Crit Care Med* 2005; 171: 722-727.

13. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, Jeffery PK. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. *Am J Respir Crit Care Med* 2007; 176: 858-864.

14. Saglani S, Mathie SA, Gregory LG, Bell MJ, Bush A, Lloyd CM. Pathophysiological features of asthma develop in parallel in house dust mite-exposed neonatal mice. *Am J Respir Cell Mol Biol* 2009; 41: 281-289.

15. Kariyawasam HH, Aizen M, Barkans J, Robinson DS, Kay AB. Remodeling and airway hyperresponsiveness but not cellular inflammation persist after allergen challenge in asthma. *Am J Respir Crit Care Med* 2007; 175: 896-904.

16. Lezmi G, Gosset P, Deschildre A, Abou-Taam R, Mahut B, Beydon N, de Blic J. Airway Remodeling in Preschool Children with Severe Recurrent Wheeze. *Am J Respir Crit Care Med* 2015; 192: 164-171.

17. Grainge CL, Lau LC, Ward JA, Dulay V, Lahiff G, Wilson S, Holgate S, Davies DE, Howarth PH. Effect of bronchoconstriction on airway remodeling in asthma. *N Engl J Med* 2011; 364: 2006-2015.

18. Kuo C, Lim S, King NJ, Bartlett NW, Walton RP, Zhu J, Glanville N, Aniscenko J, Johnston SL, Burgess JK, Black JL, Oliver BG. Rhinovirus infection induces expression of airway remodelling factors in vitro and in vivo. *Respirology* 2011; 16: 367-377.

19. Tliba O, Panettieri RA,Jr. Noncontractile functions of airway smooth muscle cells in asthma. *Annu Rev Physiol* 2009; 71: 509-535.

20. Panettieri RA,Jr, Kotlikoff MI, Gerthoffer WT, Hershenson MB, Woodruff PG, Hall IP, Banks-Schlegel S, National Heart, Lung,and Blood Institute. Airway smooth muscle in bronchial tone, inflammation, and remodeling: basic knowledge to clinical relevance *Am J Respir Crit Care Med* 2008; 177: 248-252.

21. Makinde T, Murphy RF, Agrawal DK. Immunomodulatory role of vascular endothelial growth factor and angiopoietin-1 in airway remodeling. *Curr Mol Med* 2006; 6: 831-841.

22. Berair R, Brightling CE. Asthma therapy and its effect on airway remodelling. *Drugs* 2014; 74: 1345-1369.

23. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006; 117: 1277-1284.

24. Bradding P, Holgate ST. Immunopathology and human mast cell cytokines. *Crit Rev Oncol Hematol* 1999; 31: 119-133.

25. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001; 193: 255-261.

26. Woodman L, Siddiqui S, Cruse G, Sutcliffe A, Saunders R, Kaur D, Bradding P, Brightling C. Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1 *J Immunol* 2008; 181: 5001-5007.

27. Berger P, Perng DW, Thabrew H, Compton SJ, Cairns JA, McEuen AR, Marthan R, Tunon De Lara JM, Walls AF. Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells. *J Appl Physiol (1985)* 2001; 91: 1372-1379.

28. Kaur D, Hollins F, Saunders R, Woodman L, Sutcliffe A, Cruse G, Bradding P, Brightling C. Airway smooth muscle proliferation and survival is not modulated by mast cells. *Clin Exp Allergy* 2010; 40: 279-288.

29. Naveed SU, Clements D, Jackson DJ, Philp C, Billington CK, Soomro I, Reynolds C, Harrison TW, Johnston SL, Shaw DE, Johnson SR. Matrix Metalloproteinase-1 Activation Contributes to Airway Smooth Muscle Growth and Asthma Severity. *Am J Respir Crit Care Med* 2017; 195: 1000-1009.

30. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* 2008; 8: 205-217.

31. Roth M, Zhong J, Zumkeller C, S'ng CT, Goulet S, Tamm M. The role of IgE-receptors in IgE-dependent airway smooth muscle cell remodelling. *PLoS One* 2013; 8: e56015.

32. Okumura S, Sagara H, Fukuda T, Saito H, Okayama Y. FcepsilonRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. *J Allergy Clin Immunol* 2005; 115: 272-279.

33. Wang SW, Oh CK, Cho SH, Hu G, Martin R, Demissie-Sanders S, Li K, Moyle M, Yao Z. Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts. *J Allergy Clin Immunol* 2005; 115: 287-294.

34. Shim JY, Park SW, Kim DS, Shim JW, Jung HL, Park MS. The effect of interleukin-4 and amphiregulin on the proliferation of human airway smooth muscle cells and cytokine release. *J Korean Med Sci* 2008; 23: 857-863.

35. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, Chu HW. Evidence that severe asthma can be divided pathologically into two inflammatory

subtypes with distinct physiologic and clinical characteristics *Am J Respir Crit Care Med* 1999; 160: 1001-1008.

36. Motojima S, Frigas E, Loegering DA, Gleich GJ. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro *Am Rev Respir Dis* 1989; 139: 801-805.

37. Ramos-Barbon D, Fraga-Iriso R, Brienza NS, Montero-Martinez C, Verea-Hernando H, Olivenstein R, Lemiere C, Ernst P, Hamid QA, Martin JG. T Cells localize with proliferating smooth muscle alpha-actin+ cell compartments in asthma. *Am J Respir Crit Care Med* 2010; 182: 317-324.

38. Bates JH, Martin JG. A theoretical study of the effect of airway smooth muscle orientation on bronchoconstriction. *J Appl Physiol (1985)* 1990; 69: 995-1001.

39. Mitzner W. Airway smooth muscle: the appendix of the lung *Am J Respir Crit Care Med* 2004; 169: 787-790.

40. Amrani Y, Panettieri RA. Airway smooth muscle: contraction and beyond. *Int J Biochem Cell Biol* 2003; 35: 272-276.

41. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma *Am J Respir Crit Care Med* 2003; 167: 1360-1368.

42. Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, Carter R, Wong HH, Cadbury PS, Fahy JV. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression *Am J Respir Crit Care Med* 2004; 169: 1001-1006.

43. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma *Thorax* 2007; 62: 8-15.

44. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, Burgess JK, Black JL. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001; 164: 474-477.

45. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, Ousova O, Vernejoux JM, Marthan R, Tunon-de-Lara JM, Berger P. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma *J Exp Med* 2007; 204: 3173-3181.

46. Ward JE, Harris T, Bamford T, Mast A, Pain MC, Robertson C, Smallwood D, Tran T, Wilson J, Stewart AG. Proliferation is not increased in airway myofibroblasts isolated from asthmatics. *Eur Respir J* 2008; 32: 362-371.

47. Popova AP, Bozyk PD, Bentley JK, Linn MJ, Goldsmith AM, Schumacher RE, Weiner GM, Filbrun AG, Hershenson MB. Isolation of tracheal aspirate mesenchymal stromal cells predicts bronchopulmonary dysplasia *Pediatrics* 2010; 126: e1127-33.

48. Walker N, Badri L, Wettlaufer S, Flint A, Sajjan U, Krebsbach PH, Keshamouni VG, Peters-Golden M, Lama VN. Resident tissue-specific mesenchymal progenitor cells contribute to fibrogenesis in human lung allografts *Am J Pathol* 2011; 178: 2461-2469.

49. Bentley JK, Popova AP, Bozyk PD, Linn MJ, Baek AE, Lei J, Goldsmith AM, Hershenson MB. Ovalbumin sensitization and challenge increases the number of lung cells possessing a mesenchymal stromal cell phenotype *Respir Res* 2010; 11: 127-9921-11-127.

50. Sato K, Ozaki K, Mori M, Muroi K, Ozawa K. Mesenchymal stromal cells for graft-versus-host disease : basic aspects and clinical outcomes *J Clin Exp Hematop* 2010; 50: 79-89.

51. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1994; 1: 71-81.

52. Blakaj A, Bucala R. Fibrocytes in health and disease. *Fibrogenesis Tissue Repair* 2012; 5 Suppl 1: S6.

53. Herzog EL, Bucala R. Fibrocytes in health and disease. *Exp Hematol* 2010; 38: 548-556.

54. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 2001; 166: 7556-7562.

55. Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R. Circulating fibrocytes: collagen-secreting cells of the peripheral blood. *Int J Biochem Cell Biol* 2004; 36: 598-606.

56. Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol* 2003; 171: 380-389.

57. Nihlberg K, Larsen K, Hultgardh-Nilsson A, Malmstrom A, Bjermer L, Westergren-Thorsson G. Tissue fibrocytes in patients with mild asthma: a possible link to thickness of reticular basement membrane? *Respir Res* 2006; 7: 50.

58. Wang C-, Huang C-, Lin H-, Lee K-, Lin S-, Liu C-, Huang K-, Ko Y-, Chung KF, Kuo H-. Increased Circulating Fibrocytes in Asthma with Chronic Airflow Obstruction *American Journal of Respiratory and Critical Care Medicine* 2008; 178: 583 <last_page> 591.

59. Wang CH, Huang CD, Lin HC, Huang TT, Lee KY, Lo YL, Lin SM, Chung KF, Kuo HP. Increased activation of fibrocytes in patients with chronic obstructive asthma through an epidermal growth factor receptor-dependent pathway. *J Allergy Clin Immunol* 2012; 129: 1367-1376.

60. Saunders R, Siddiqui S, Kaur D, Doe C, Sutcliffe A, Hollins F, Bradding P, Wardlaw A, Brightling CE. Fibrocyte localization to the airway smooth muscle is a feature of asthma *J Allergy Clin Immunol* 2009; 123: 376-384.

61. Berair R, Hollins F, Brightling C. Airway smooth muscle hypercontractility in asthma *J Allergy (Cairo)* 2013; 2013: 185971.

62. Sutcliffe A, Hollins F, Gomez E, Saunders R, Doe C, Cooke M, Challiss RA, Brightling CE. Increased nicotinamide adenine dinucleotide phosphate oxidase 4 expression mediates intrinsic airway smooth muscle hypercontractility in asthma *Am J Respir Crit Care Med* 2012; 185: 267-274.

63. Matsumoto H, Moir LM, Oliver BG, Burgess JK, Roth M, Black JL, McParland BE. Comparison of gel contraction mediated by airway smooth muscle cells from patients with and without asthma *Thorax* 2007; 62: 848-854.

64. Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JPT, Lee TH. Ca2+ homeostasis and structural and functional remodelling of airway smooth muscle in asthma *Thorax* 2010; 65: 547-552.

65. Chiba Y, Takada Y, Miyamoto S, MitsuiSaito M, Karaki H, Misawa M. Augmented acetylcholine-induced, Rho-mediated Ca2+ sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats *Br J Pharmacol* 1999; 127: 597-600.

66. Gunst SJ, Panettieri RA,Jr. Point: alterations in airway smooth muscle phenotype do/do not cause airway hyperresponsiveness in asthma. *J Appl Physiol (1985)* 2012; 113: 837-839.

67. Johnson PR, Black JL, Carlin S, Ge Q, Underwood PA. The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. *Am J Respir Crit Care Med* 2000; 162: 2145-2151.

68. Damera G, Tliba O, Panettieri RA,Jr. Airway smooth muscle as an immunomodulatory cell. *Pulm Pharmacol Ther* 2009; 22: 353-359.

69. Damera G, Panettieri RA,Jr. Does airway smooth muscle express an inflammatory phenotype in asthma? *Br J Pharmacol* 2011; 163: 68-80.

70. Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997; 156: 951-958.

71. Roberts CR, Burke AK. Remodelling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. *Can Respir J* 1998; 5: 48-50.

72. Slats AM, Janssen K, van Schadewijk A, van der Plas DT, Schot R, van den Aardweg JG, de Jongste JC, Hiemstra PS, Mauad T, Rabe KF, Sterk PJ. Expression of smooth muscle and extracellular matrix proteins in relation to airway function in asthma. *J Allergy Clin Immunol* 2008; 121: 1196-1202.

73. Milanese M, Crimi E, Scordamaglia A, Riccio A, Pellegrino R, Canonica GW, Brusasco V. On the functional consequences of bronchial basement membrane thickening. *J Appl Physiol (1985)* 2001; 91: 1035-1040.

74. Kasahara K, Shiba K, Ozawa T, Okuda K, Adachi M. Correlation between the bronchial subepithelial layer and whole airway wall thickness in patients with asthma. *Thorax* 2002; 57: 242-246.

75. Shiba K, Kasahara K, Nakajima H, Adachi M. Structural changes of the airway wall impair respiratory function, even in mild asthma. *Chest* 2002; 122: 1622-1626.

76. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, McElwain K, McElwain S, Friedman S, Broide DH. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest* 2004; 113: 551-560.

77. McMillan SJ, Xanthou G, Lloyd CM. Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway. *J Immunol* 2005; 174: 5774-5780.

78. Royce SG, Cheng V, Samuel CS, Tang ML. The regulation of fibrosis in airway remodeling in asthma. *Mol Cell Endocrinol* 2012; 351: 167-175.

79. Salvato G. Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. *Thorax* 2001; 56: 902-906.

80. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. *Chest* 2005; 127: 965-972.

81. Siddiqui S, Sutcliffe A, Shikotra A, Woodman L, Doe C, McKenna S, Wardlaw A, Bradding P, Pavord I, Brightling C. Vascular remodeling is a feature of asthma and nonasthmatic eosinophilic bronchitis. *J Allergy Clin Immunol* 2007; 120: 813-819.

82. Feltis BN, Wignarajah D, Reid DW, Ward C, Harding R, Walters EH. Effects of inhaled fluticasone on angiogenesis and vascular endothelial growth factor in asthma. *Thorax* 2007; 62: 314-319.

83. Goldie RG, Pedersen KE. Mechanisms of increased airway microvascular permeability: role in airway inflammation and obstruction. *Clin Exp Pharmacol Physiol* 1995; 22: 387-396.

84. Tam A, Wadsworth S, Dorscheid D, Man SF, Sin DD. The airway epithelium: more than just a structural barrier. *Ther Adv Respir Dis* 2011; 5: 255-273.

85. Gern JE. Rhinovirus and the initiation of asthma. *Curr Opin Allergy Clin Immunol* 2009; 9: 73-78.

86. Ahanchian H, Jones CM, Chen YS, Sly PD. Respiratory viral infections in children with asthma: do they matter and can we prevent them? *BMC Pediatr* 2012; 12: 147-2431-12-147.

87. Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, Ravichandran KS. Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. *Nature* 2013; 493: 547-551.

88. Ahdieh M, Vandenbos T, Youakim A. Lung epithelial barrier function and wound healing are decreased by IL-4 and IL-13 and enhanced by IFN-gamma. *Am J Physiol Cell Physiol* 2001; 281: C2029-38.

89. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation *Can J Physiol Pharmacol* 2008; 86: 105-112.

90. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi HM, Vernon-Wilson E, Sammut D, Bedke N, Cremin C, Sones J, Djukanovic R, Howarth PH, Collins JE, Holgate ST, Monk P, Davies DE. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol* 2011; 128: 549-56.e1-12.

91. Jiang A, Bloom O, Ono S, Cui W, Unternaehrer J, Jiang S, Whitney JA, Connolly J, Banchereau J, Mellman I. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 2007; 27: 610-624.

92. Bucchieri F, Puddicombe SM, Lordan JL, Richter A, Buchanan D, Wilson SJ, Ward J, Zummo G, Howarth PH, Djukanovic R, Holgate ST, Davies DE. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol* 2002; 27: 179-185.

93. Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM. Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. *Am J Respir Crit Care Med* 2006; 174: 1110-1118.

94. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000; 14: 1362-1374.

95. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr Opin Allergy Clin Immunol* 2012; 12: 53-59.

96. Heijink IH, Postma DS, Noordhoek JA, Broekema M, Kapus A. House dust mite-promoted epithelial-to-mesenchymal transition in human bronchial epithelium *Am J Respir Cell Mol Biol* 2010; 42: 69-79.

97. Torrego A, Hew M, Oates T, Sukkar M, Fan Chung K. Expression and activation of TGF-beta isoforms in acute allergen-induced remodelling in asthma. *Thorax* 2007; 62: 307-313.

98. Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, Argentieri R, Kicic A, Stick SM, Bai TR, Knight DA. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1 *Am J Respir Crit Care Med* 2009; 180: 122-133.

99. Johnson JR, Roos A, Berg T, Nord M, Fuxe J. Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways *PLoS One* 2011; 6: e16175.

100. Yasukawa A, Hosoki K, Toda M, Miyake Y, Matsushima Y, Matsumoto T, Boveda-Ruiz D, Gil-Bernabe P, Nagao M, Sugimoto M, Hiraguchi Y, Tokuda R, Naito M, Takagi T, D'Alessandro-Gabazza CN, Suga S, Kobayashi T, Fujisawa T, Taguchi O, Gabazza EC. Eosinophils promote epithelial to mesenchymal transition of bronchial epithelial cells. *PLoS One* 2013; 8: e64281.

101. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006; 86: 245-278.

102. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* 2008; 70: 459-486.

103. DUNNILL MS. The pathology of asthma, with special reference to changes in the bronchial mucosa. *J Clin Pathol* 1960; 13: 27-33.

104. Ulrik CS. Outcome of asthma: longitudinal changes in lung function. *Eur Respir J* 1999; 13: 904-918.

105. Jinnai M, Niimi A, Ueda T, Matsuoka H, Takemura M, Yamaguchi M, Otsuka K, Oguma T, Takeda T, Ito I, Matsumoto H, Mishima M. Induced sputum concentrations of mucin in patients with asthma and chronic cough. *Chest* 2010; 137: 1122-1129.

106. Bry K, Whitsett JA, Lappalainen U. IL-1beta disrupts postnatal lung morphogenesis in the mouse. *Am J Respir Cell Mol Biol* 2007; 36: 32-42.

107. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, Tam DC, Nadel JA. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2001; 280: L134-40.

108. Thomas B, Rutman A, Hirst RA, Haldar P, Wardlaw AJ, Bankart J, Brightling CE, O'Callaghan C. Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma. *J Allergy Clin Immunol* 2010; 126: 722-729.e2.

109. McParland BE, Macklem PT, Pare PD. Airway wall remodeling: friend or foe? *J Appl Physiol* 2003; 95: 426-434.

110. Royce SG, Tan L, Koek AA, Tang ML. Effect of extracellular matrix composition on airway epithelial cell and fibroblast structure: implications for airway remodeling in asthma. *Ann Allergy Asthma Immunol* 2009; 102: 238-246.

111. Bergeron C, Tulic MK, Hamid Q. Tools used to measure airway remodelling in research. *Eur Respir J* 2007; 29: 596-604.

112. Woodruff PG, Innes AL. Quantitative morphology using bronchial biopsies *Eur Respir Rev* 2006; 15: 157-161.

113. Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998; 12: 811-815.

114. Veraldi KL, Gibson BT, Yasuoka H, Myerburg MM, Kelly EA, Balzar S, Jarjour NN, Pilewski JM, Wenzel SE, Feghali-Bostwick CA. Role of insulin-like growth factor binding protein-3 in allergic airway remodeling. *Am J Respir Crit Care Med* 2009; 180: 611-617.

115. Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, Howarth PH. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 1997; 156: 642-647.

116. Zietkowski Z, Skiepko R, Tomasiak MM, Bodzenta-Lukaszyk A. Endothelin-1 in exhaled breath condensate of stable and unstable asthma patients. *Respir Med* 2008; 102: 470-474.

117. Kanazawa H, Yoshikawa T. Up-regulation of thrombin activity induced by vascular endothelial growth factor in asthmatic airways. *Chest* 2007; 132: 1169-1174.

118. Coxson HO, Lam S. Quantitative assessment of the airway wall using computed tomography and optical coherence tomography. *Proc Am Thorac Soc* 2009; 6: 439-443.

119. Gutierrez-Chico JL, Alegria-Barrero E, Teijeiro-Mestre R, Chan PH, Tsujioka H, de Silva R, Viceconte N, Lindsay A, Patterson T, Foin N, Akasaka T, di Mario C. Optical coherence tomography: from research to practice. *Eur Heart J Cardiovasc Imaging* 2012; 13: 370-384.

120. Coxson HO, Quiney B, Sin DD, Xing L, McWilliams AM, Mayo JR, Lam S. Airway wall thickness assessed using computed tomography and optical coherence tomography. *Am J Respir Crit Care Med* 2008; 177: 1201-1206.

121. Lam S, Lee AMD, Lane P, Ohtani K, MacAulay C, Varfolomeva N, Hui L, Coxson HO, Fitzgerald J. Optical Coherence Tomography Imaging Of Asthmatic Airways Before And After Thermoplasty. *Am J Respir Crit Care Med* 2013; 187: A6014.

122. Mould RF. The early history of x-ray diagnosis with emphasis on the contributions of physics 1895-1915. *Phys Med Biol* 1995; 40: 1741-1787.

123. Liguori C, Frauenfelder G, Massaroni C, Saccomandi P, Giurazza F, Pitocco F, Marano R, Schena E. Emerging clinical applications of computed tomography. *Med Devices (Auckl)* 2015; 8: 265-278.

124. Walker C, Gupta S, Hartley R, Brightling CE. Computed tomography scans in severe asthma: utility and clinical implications. *Curr Opin Pulm Med* 2012; 18: 42-47.

125. Bumbacea D, Campbell D, Nguyen L, Carr D, Barnes PJ, Robinson D, Chung KF. Parameters associated with persistent airflow obstruction in chronic severe asthma. *Eur Respir J* 2004; 24: 122-128.

126. Gupta S, Siddiqui S, Haldar P, Raj JV, Entwisle JJ, Wardlaw AJ, Bradding P, Pavord ID, Green RH, Brightling CE. Qualitative analysis of high-resolution CT scans in severe asthma. *Chest* 2009; 136: 1521-1528.

127. Aysola RS, Hoffman EA, Gierada D, Wenzel S, Cook-Granroth J, Tarsi J, Zheng J, Schechtman KB, Ramkumar TP, Cochran R, Xueping E, Christie C, Newell J, Fain S, Altes TA, Castro M. Airway remodeling measured by multidetector CT is increased in severe asthma and correlates with pathology. *Chest* 2008; 134: 1183-1191.

128. Siddiqui S, Gupta S, Cruse G, Haldar P, Entwisle J, Mcdonald S, Whithers PJ, Hainsworth SV, Coxson HO, Brightling C. Airway wall geometry in asthma and nonasthmatic eosinophilic bronchitis. *Allergy* 2009; 64: 951-958.

129. Saglani S, Papaioannou G, Khoo L, Ujita M, Jeffery PK, Owens C, Hansell DM, Payne DN, Bush A. Can HRCT be used as a marker of airway remodelling in children with difficult asthma? *Respir Res* 2006; 7: 46.

130. Gono H, Fujimoto K, Kawakami S, Kubo K. Evaluation of airway wall thickness and air trapping by HRCT in asymptomatic asthma. *Eur Respir J* 2003; 22: 965-971.

131. Busacker A, Newell JD,Jr, Keefe T, Hoffman EA, Granroth JC, Castro M, Fain S, Wenzel S. A multivariate analysis of risk factors for the air-trapping asthmatic phenotype as measured by quantitative CT analysis. *Chest* 2009; 135: 48-56.

132. Ueda T, Niimi A, Matsumoto H, Takemura M, Hirai T, Yamaguchi M, Matsuoka H, Jinnai M, Muro S, Chin K, Mishima M. Role of small airways in asthma: investigation using high-resolution computed tomography. *J Allergy Clin Immunol* 2006; 118: 1019-1025.

133. de Blic J, Tillie-Leblond I, Emond S, Mahut B, Dang Duy TL, Scheinmann P. High-resolution computed tomography scan and airway remodeling in children with severe asthma. *J Allergy Clin Immunol* 2005; 116: 750-754.

134. Montaudon M, Lederlin M, Reich S, Begueret H, Tunon-de-Lara JM, Marthan R, Berger P, Laurent F. Bronchial measurements in patients with asthma: comparison of quantitative thin-section CT findings with those in healthy subjects and correlation with pathologic findings. *Radiology* 2009; 253: 844-853.

135. Kasahara K, Shiba K, Ozawa T, Okuda K, Adachi M. Correlation between the bronchial subepithelial layer and whole airway wall thickness in patients with asthma. *Thorax* 2002; 57: 242-246.

136. Furuta GT, Atkins FD, Lee NA, Lee JJ. Changing roles of eosinophils in health and disease. *Ann Allergy Asthma Immunol* 2014; 113: 3-8.

137. GINA Report, Global Strategy for Asthma Management and Prevention. *http://www ginasthma org;* 2012.

138. Heitzer MD, Wolf IM, Sanchez ER, Witchel SF, DeFranco DB. Glucocorticoid receptor physiology. *Rev Endocr Metab Disord* 2007; 8: 321-330.

139. Alangari AA. Genomic and non-genomic actions of glucocorticoids in asthma. *Ann Thorac Med* 2010; 5: 133-139.

140. Lowenberg M, Verhaar AP, van den Brink GR, Hommes DW. Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. *Trends Mol Med* 2007; 13: 158-163.

141. Zhou J, Liu DF, Liu C, Kang ZM, Shen XH, Chen YZ, Xu T, Jiang CL. Glucocorticoids inhibit degranulation of mast cells in allergic asthma via nongenomic mechanism. *Allergy* 2008; 63: 1177-1185.

142. Kagoshima M, Wilcke T, Ito K, Tsaprouni L, Barnes PJ, Punchard N, Adcock IM. Glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation. *Eur J Pharmacol* 2001; 429: 327-334.

143. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schutz G. DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 1998; 93: 531-541.

144. Reichardt HM, Tuckermann JP, Gottlicher M, Vujic M, Weih F, Angel P, Herrlich P, Schutz G. Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *EMBO J* 2001; 20: 7168-7173.

145. Freishtat RJ, Nagaraju K, Jusko W, Hoffman EP. Glucocorticoid efficacy in asthma: is improved tissue remodeling upstream of anti-inflammation. *J Investig Med* 2010; 58: 19-22.

146. Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ. Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. *Chest* 2002; 122: 1543-1552.

147. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999; 104: 356-363.

148. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, Walters EH. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 2002; 57: 309-316.

149. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 1999; 159: 1043-1051.

150. Chetta A, Zanini A, Foresi A, Del Donno M, Castagnaro A, D'Ippolito R, Baraldo S, Testi R, Saetta M, Olivieri D. Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. *Am J Respir Crit Care Med* 2003; 167: 751-757.

151. Trigg CJ, Manolitsas ND, Wang J, Calderon MA, McAulay A, Jordan SE, Herdman MJ, Jhalli N, Duddle JM, Hamilton SA. Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma. *Am J Respir Crit Care Med* 1994; 150: 17-22.

152. Olivieri D, Chetta A, Del Donno M, Bertorelli G, Casalini A, Pesci A, Testi R, Foresi A. Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study. *Am J Respir Crit Care Med* 1997; 155: 1864-1871.

153. Hoshino M, Nakamura Y, Sim JJ, Yamashiro Y, Uchida K, Hosaka K, Isogai S. Inhaled corticosteroid reduced lamina reticularis of the basement membrane by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma. *Clin Exp Allergy* 1998; 28: 568-577.

154. Lundgren R, Soderberg M, Horstedt P, Stenling R. Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur Respir J* 1988; 1: 883-889.

155. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, Johansson SA. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. *Am Rev Respir Dis* 1992; 145: 890-899.

156. Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, Chakir J. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* 2000; 162: 1308-1313.

157. Bergeron C, Hauber HP, Gotfried M, Newman K, Dhanda R, Servi RJ, Ludwig MS, Hamid Q. Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: effect of hydrofluoroalkane-flunisolide. *J Allergy Clin Immunol* 2005; 116: 983-989.

158. Dorscheid DR, Low E, Conforti A, Shifrin S, Sperling AI, White SR. Corticosteroid-induced apoptosis in mouse airway epithelium: effect in normal airways and after allergen-induced airway inflammation. *J Allergy Clin Immunol* 2003; 111: 360-366.

159. White SR, Dorscheid DR. Corticosteroid-induced apoptosis of airway epithelium: a potential mechanism for chronic airway epithelial damage in asthma. *Chest* 2002; 122: 278S-284S.

160. Dorscheid DR, Wojcik KR, Sun S, Marroquin B, White SR. Apoptosis of airway epithelial cells induced by corticosteroids. *Am J Respir Crit Care Med* 2001; 164: 1939-1947.

161. Erjefalt JS, Erjefalt I, Sundler F, Persson CG. Effects of topical budesonide on epithelial restitution in vivo in guinea pig trachea. *Thorax* 1995; 50: 785-792.

162. Wadsworth SJ, Nijmeh HS, Hall IP. Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model. *J Clin Immunol* 2006; 26: 376-387.

163. Liu J, Zhang M, Niu C, Luo Z, Dai J, Wang L, Liu E, Fu Z. Dexamethasone inhibits repair of human airway epithelial cells mediated by glucocorticoid-induced leucine zipper (GILZ). *PLoS One* 2013; 8: e60705.

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

165. Stewart AG, Fernandes D, Tomlinson PR. The effect of glucocorticoids on proliferation of human cultured airway smooth muscle. *Br J Pharmacol* 1995; 116: 3219-3226.

166. Cha HH, Cram EJ, Wang EC, Huang AJ, Kasler HG, Firestone GL. Glucocorticoids stimulate p21 gene expression by targeting multiple transcriptional elements within a steroid responsive region of the p21waf1/cip1 promoter in rat hepatoma cells. *J Biol Chem* 1998; 273: 1998-2007.

167. Fernandes D, Guida E, Koutsoubos V, Harris T, Vadiveloo P, Wilson JW, Stewart AG. Glucocorticoids inhibit proliferation, cyclin D1 expression, and retinoblastoma protein phosphorylation, but not activity of the extracellular-regulated kinases in human cultured airway smooth muscle. *Am J Respir Cell Mol Biol* 1999; 21: 77-88.

168. Roth M, Johnson PR, Borger P, Bihl MP, Rudiger JJ, King GG, Ge Q, Hostettler K, Burgess JK, Black JL, Tamm M. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004; 351: 560-574.

169. Cazes E, Giron-Michel J, Baouz S, Doucet C, Cagnoni F, Oddera S, Korner M, Dasic G, Testi R, Azzarone B, Canonica GW. Novel anti-inflammatory effects of the inhaled corticosteroid fluticasone propionate during lung myofibroblastic differentiation. *J Immunol* 2001; 167: 5329-5337.

170. Leung SY, Eynott P, Nath P, Chung KF. Effects of ciclesonide and fluticasone propionate on allergen-induced airway inflammation and remodeling features. *J Allergy Clin Immunol* 2005; 115: 989-996.

171. Chetta A, Olivieri D. Role of Inhaled Steroids in Vascular Airway Remodelling in Asthma and COPD. *Int J Endocrinol* 2012; 2012: 397693.

172. Orsida BE, Li X, Hickey B, Thien F, Wilson JW, Walters EH. Vascularity in asthmatic airways: relation to inhaled steroid dose. *Thorax* 1999; 54: 289-295.

173. Hoshino M, Takahashi M, Takai Y, Sim J, Aoike N. Inhaled corticosteroids decrease vascularity of the bronchial mucosa in patients with asthma. *Clin Exp Allergy* 2001; 31: 722-730.

174. Feltis BN, Wignarajah D, Reid DW, Ward C, Harding R, Walters EH. Effects of inhaled fluticasone on angiogenesis and vascular endothelial growth factor in asthma. *Thorax* 2007; 62: 314-319.

175. Orsida BE, Ward C, Li X, Bish R, Wilson JW, Thien F, Walters EH. Effect of a long-acting beta2-agonist over three months on airway wall vascular remodeling in asthma. *Am J Respir Crit Care Med* 2001; 164: 117-121.

176. Nelson HS, Weiss ST, Bleecker ER, Yancey SW, Dorinsky PM, SMART Study Group. The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol. *Chest* 2006; 129: 15-26.

177. Todorova L, Gurcan E, Miller-Larsson A, Westergren-Thorsson G. Lung fibroblast proteoglycan production induced by serum is inhibited by budesonide and formoterol. *Am J Respir Cell Mol Biol* 2006; 34: 92-100.

178. Todorova L, Gurcan E, Westergren-Thorsson G, Miller-Larsson A. Budesonide/formoterol effects on metalloproteolytic balance in TGFbeta-activated human lung fibroblasts. *Respir Med* 2009; 103: 1755-1763.

179. Peters-Golden M, Henderson WR,Jr. Leukotrienes. *N Engl J Med* 2007; 357: 1841-1854.

180. Hallstrand TS, Henderson WR,Jr. An update on the role of leukotrienes in asthma. *Curr Opin Allergy Clin Immunol* 2010; 10: 60-66.

181. Asanuma F, Kuwabara K, Arimura A, Furue Y, Fleisch JH, Hori Y. Effects of leukotriene B4 receptor antagonist, LY293111Na, on antigen-induced bronchial hyperresponsiveness and leukocyte infiltration in sensitized guinea pigs. *Inflamm Res* 2001; 50: 136-141.

182. Tager AM, Bromley SK, Medoff BD, Islam SA, Bercury SD, Friedrich EB, Carafone AD, Gerszten RE, Luster AD. Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nat Immunol* 2003; 4: 982-990.

183. Phan SH, McGarry BM, Loeffler KM, Kunkel SL. Binding of leukotriene C4 to rat lung fibroblasts and stimulation of collagen synthesis in vitro. *Biochemistry* 1988; 27: 2846-2853.

184. Ozaki T, Hayashi H, Tani K, Ogushi F, Yasuoka S, Ogura T. Neutrophil chemotactic factors in the respiratory tract of patients with chronic airway diseases or idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 1992; 145: 85-91.

185. Panettieri RA, Tan EM, Ciocca V, Luttmann MA, Leonard TB, Hay DW. Effects of LTD4 on human airway smooth muscle cell proliferation, matrix expression, and

contraction In vitro: differential sensitivity to cysteinyl leukotriene receptor antagonists. *Am J Respir Cell Mol Biol* 1998; 19: 453-461.

186. Cohen P, Noveral JP, Bhala A, Nunn SE, Herrick DJ, Grunstein MM. Leukotriene D4 facilitates airway smooth muscle cell proliferation via modulation of the IGF axis. *Am J Physiol* 1995; 269: L151-7.

187. Chauhan BF, Ducharme FM. Anti-leukotriene agents compared to inhaled corticosteroids in the management of recurrent and/or chronic asthma in adults and children. *Cochrane Database Syst Rev* 2012; 5: CD002314.

188. Bousquet J, Demoly P, Humbert M. Montelukast in guidelines and beyond. *Adv Ther* 2009; 26: 575-587.

189. Joos S, Miksch A, Szecsenyi J, Wieseler B, Grouven U, Kaiser T, Schneider A. Montelukast as add-on therapy to inhaled corticosteroids in the treatment of mild to moderate asthma: a systematic review. *Thorax* 2008; 63: 453-462.

190. Muz MH, Deveci F, Bulut Y, Ilhan N, Yekeler H, Turgut T. The effects of low dose leukotriene receptor antagonist therapy on airway remodeling and cysteinyl leukotriene expression in a mouse asthma model. *Exp Mol Med* 2006; 38: 109-118.

191. Henderson WR,Jr, Tang LO, Chu SJ, Tsao SM, Chiang GK, Jones F, Jonas M, Pae C, Wang H, Chi EY. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *Am J Respir Crit Care Med* 2002; 165: 108-116.

192. Henderson WR,Jr, Chiang GK, Tien YT, Chi EY. Reversal of allergen-induced airway remodeling by CysLT1 receptor blockade. *Am J Respir Crit Care Med* 2006; 173: 718-728.

193. Gobbato NB, de Souza FC, Fumagalli SB, Lopes FD, Prado CM, Martins MA, Tiberio Ide F, Leick EA. Antileukotriene reverts the early effects of inflammatory response of distal parenchyma in experimental chronic allergic inflammation. *Biomed Res Int* 2013; 2013: 523761.

194. Rabe KF, Magnussen H, Dent G. Theophylline and selective PDE inhibitors as bronchodilators and smooth muscle relaxants. *Eur Respir J* 1995; 8: 637-642.

195. Lagente V, Pruniaux MP, Junien JL, Moodley I. Modulation of cytokine-induced eosinophil infiltration by phosphodiesterase inhibitors. *Am J Respir Crit Care Med* 1995; 151: 1720-1724.

196. Sullivan P, Bekir S, Jaffar Z, Page C, Jeffery P, Costello J. Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. *Lancet* 1994; 343: 1006-1008.

197. Horiguchi T, Tachikawa S, Kasahara J, Doi M, Shiga M, Miyazaki J, Sasaki Y, Hirose M, Imazu M. Suppression of airway inflammation by theophylline in adult bronchial asthma. *Respiration* 1999; 66: 124-127.

198. Hendeles L, Harman E, Huang D, O'Brien R, Blake K, Delafuente J. Theophylline attenuation of airway responses to allergen: comparison with cromolyn metered-dose inhaler. *J Allergy Clin Immunol* 1995; 95: 505-514.

199. Magnussen H, Reuss G, Jorres R. Theophylline has a dose-related effect on the airway response to inhaled histamine and methacholine in asthmatics. *Am Rev Respir Dis* 1987; 136: 1163-1167.

200. Barnes PJ. Histone deacetylase-2 and airway disease. *Ther Adv Respir Dis* 2009; 3: 235-243.

201. Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res* 2006; 7: 73.

202. Matthiesen S, Bahulayan A, Kempkens S, Haag S, Fuhrmann M, Stichnote C, Juergens UR, Racke K. Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. *Am J Respir Cell Mol Biol* 2006; 35: 621-627.

203. Gosens R, Nelemans SA, Grootte Bromhaar MM, McKay S, Zaagsma J, Meurs H. Muscarinic M3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *Am J Respir Cell Mol Biol* 2003; 28: 257-262.

204. Oenema TA, Smit M, Smedinga L, Racke K, Halayko AJ, Meurs H, Gosens R. Muscarinic receptor stimulation augments TGF-beta1-induced contractile protein expression by airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2012; 303: L589-97.

205. Kerstjens HA, Engel M, Dahl R, Paggiaro P, Beck E, Vandewalker M, Sigmund R, Seibold W, Moroni-Zentgraf P, Bateman ED. Tiotropium in asthma poorly controlled with standard combination therapy. *N Engl J Med* 2012; 367: 1198-1207.

206. Kerstjens HA, Disse B, Schroder-Babo W, Bantje TA, Gahlemann M, Sigmund R, Engel M, van Noord JA. Tiotropium improves lung function in patients with severe uncontrolled asthma: a randomized controlled trial. *J Allergy Clin Immunol* 2011; 128: 308-314.

207. Peters SP, Kunselman SJ, Icitovic N, Moore WC, Pascual R, Ameredes BT, Boushey HA, Calhoun WJ, Castro M, Cherniack RM, Craig T, Denlinger L, Engle LL, DiMango EA, Fahy JV, Israel E, Jarjour N, Kazani SD, Kraft M, Lazarus SC, Lemanske RF,Jr, Lugogo N, Martin RJ, Meyers DA, Ramsdell J, Sorkness CA, Sutherland ER, Szefler SJ, Wasserman SI, Walter MJ, Wechsler ME, Chinchilli VM, Bleecker ER, National Heart, Lung, and Blood Institute Asthma Clinical Research Network. Tiotropium bromide step-up therapy for adults with uncontrolled asthma. *N Engl J Med* 2010; 363: 1715-1726.

208. Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med* 2005; 171: 1096-1102.

209. Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur Respir J* 2007; 30: 653-661.

210. Ohta S, Oda N, Yokoe T, Tanaka A, Yamamoto Y, Watanabe Y, Minoguchi K, Ohnishi T, Hirose T, Nagase H, Ohta K, Adachi M. Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma. *Clin Exp Allergy* 2010; 40: 1266-1275.

211. Haag S, Matthiesen S, Juergens UR, Racke K. Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts. *Eur Respir J* 2008; 32: 555-562.

212. Iesato K, Tatsumi K, Saito K, Ogasawara T, Sakao S, Tada Y, Kasahara Y, Kurosu K, Tanabe N, Takiguchi Y, Kuriyama T, Shirasawa H. Tiotropium bromide attenuates respiratory syncytial virus replication in epithelial cells. *Respiration* 2008; 76: 434-441.

213. Zieg G, Lack G, Harbeck RJ, Gelfand EW, Leung DY. In vivo effects of glucocorticoids on IgE production. *J Allergy Clin Immunol* 1994; 94: 222-230.

214. Strunk RC, Bloomberg GR. Omalizumab for asthma *N Engl J Med* 2006; 354: 2689-2695.

215. Holgate ST, Chuchalin AG, Hebert J, Lotvall J, Persson GB, Chung KF, Bousquet J, Kerstjens HA, Fox H, Thirlwell J, Cioppa GD, Omalizumab 011 International Study Group. Efficacy and safety of a recombinant anti-immunoglobulin E antibody (omalizumab) in severe allergic asthma. *Clin Exp Allergy* 2004; 34: 632-638.

216. Djukanovic R, Wilson SJ, Kraft M, Jarjour NN, Steel M, Chung KF, Bao W, Fowler-Taylor A, Matthews J, Busse WW, Holgate ST, Fahy JV. Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. *Am J Respir Crit Care Med* 2004; 170: 583-593.

217. Fahy JV, Fleming HE, Wong HH, Liu JT, Su JQ, Reimann J, Fick RB,Jr, Boushey HA. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 155: 1828-1834.

218. MacGlashan DW,Jr, Bochner BS, Adelman DC, Jardieu PM, Togias A, McKenzie-White J, Sterbinsky SA, Hamilton RG, Lichtenstein LM. Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol* 1997; 158: 1438-1445.

219. Prussin C, Griffith DT, Boesel KM, Lin H, Foster B, Casale TB. Omalizumab treatment downregulates dendritic cell FcepsilonRI expression. *J Allergy Clin Immunol* 2003; 112: 1147-1154.

220. Boulet LP, Chapman KR, Cote J, Kalra S, Bhagat R, Swystun VA, Laviolette M, Cleland LD, Deschesnes F, Su JQ, DeVault A, Fick RB,Jr, Cockcroft DW. Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response. *Am J Respir Crit Care Med* 1997; 155: 1835-1840.

221. Hoshino M, Ohtawa J. Effects of adding omalizumab, an anti-immunoglobulin E antibody, on airway wall thickening in asthma. *Respiration* 2012; 83: 520-528.

222. Riccio AM, Dal Negro RW, Micheletto C, De Ferrari L, Folli C, Chiappori A, Canonica GW. Omalizumab modulates bronchial reticular basement membrane thickness and eosinophil infiltration in severe persistent allergic asthma patients. *Int J Immunopathol Pharmacol* 2012; 25: 475-484.

223. Rosenwasser LJ, Meng J. Anti-CD23. *Clin Rev Allergy Immunol* 2005; 29: 61-72.

224. Berair R, Pavord ID. Rationale and Clinical Results of Inhibiting Interleukin-5 for the Treatment of Severe Asthma *Curr Allergy Asthma Rep* 2013.

225. Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L, Robinson D, Wenzel S, Busse W, Hansel TT, Barnes NC, International Mepolizumab

Study Group. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma *Am J Respir Crit Care Med* 2007; 176: 1062-1071.

226. Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway *Am J Respir Crit Care Med* 2003; 167: 199-204.

227. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, Marshall RP, Bradding P, Green RH, Wardlaw AJ, Pavord ID. Mepolizumab and exacerbations of refractory eosinophilic asthma *N Engl J Med* 2009; 360: 973-984.

228. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, Hargreave FE, O'Byrne PM. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia *N Engl J Med* 2009; 360: 985-993.

229. Pavord ID, Korn S, Howarth P, Bleecker ER, Buhl R, Keene ON, Ortega H, Chanez P. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial *Lancet* 2012; 380: 651-659.

230. Liu Y, Zhang S, Li DW, Jiang SJ. Efficacy of anti-interleukin-5 therapy with mepolizumab in patients with asthma: a meta-analysis of randomized placebo-controlled trials *PLoS One* 2013; 8: e59872.

231. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, Barnes PJ. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response *Lancet* 2000; 356: 2144-2148.

232. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model *J Exp Med* 1996; 183: 195-201.

233. Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwara D, Takatsu K, Delespesse G, Nagai H. Role of interleukin-5 and eosinophils in allergen-induced airway remodeling in mice *Am J Respir Cell Mol Biol* 2004; 31: 62-68.

234. Haldar P, Brightling CE, Singapuri A, Hargadon B, Gupta S, Monteiro W, Bradding P, Green RH, Wardlaw AJ, Ortega H, Pavord ID. Outcomes after cessation of mepolizumab therapy in severe eosinophilic asthma: A 12-month follow-up analysis. *J Allergy Clin Immunol* 2014; 133: 921-923.

235. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282: 2258-2261.

236. Yang G, Volk A, Petley T, Emmell E, Giles-Komar J, Shang X, Li J, Das AM, Shealy D, Griswold DE, Li L. Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. *Cytokine* 2004; 28: 224-232.

237. Tomlinson KL, Davies GC, Sutton DJ, Palframan RT. Neutralisation of interleukin-13 in mice prevents airway pathology caused by chronic exposure to house dust mite. *PLoS One* 2010; 5: 10.1371/journal.pone.0013136.

238. Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, Hogaboam CM. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J Immunol* 2001; 166: 5219-5224.

239. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, Mosesova S, Eisner MD, Bohen SP, Matthews JG. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011; 365: 1088-1098.

240. Piper E, Brightling C, Niven R, Oh C, Faggioni R, Poon K, She D, Kell C, May RD, Geba GP, Molfino NA. A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. *Eur Respir J* 2013; 41: 330-338.

241. Bergeron C, Page N, Barbeau B, Chakir J. Interleukin-4 promotes airway remodeling in asthma: regulation of procollagen I (alpha1) gene by interleukin-4. *Chest* 2003; 123: 424S.

242. Gavett SH, O'Hearn DJ, Karp CL, Patel EA, Schofield BH, Finkelman FD, Wills-Karp M. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* 1997; 272: L253-61.

243. Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, Wang L, Kirkesseli S, Rocklin R, Bock B, Hamilton J, Ming JE, Radin A, Stahl N, Yancopoulos GD, Graham N, Pirozzi G. Dupilumab in persistent asthma with elevated eosinophil levels. *N Engl J Med* 2013; 368: 2455-2466.

244. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem* 2003; 278: 17036-17043.

245. Inoue D, Numasaki M, Watanabe M, Kubo H, Sasaki T, Yasuda H, Yamaya M, Sasaki H. IL-17A promotes the growth of airway epithelial cells through ERK-dependent signaling pathway. *Biochem Biophys Res Commun* 2006; 347: 852-858.

246. Busse WW, Holgate S, Kerwin E, Chon Y, Feng J, Lin J, Lin SL. Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *Am J Respir Crit Care Med* 2013; 188: 1294-1302.

247. Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W, Beckett P, Al Ali M, Chauhan A, Wilson SJ, Reynolds A, Davies DE, Holgate ST. Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005; 60: 1012-1018.

248. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, Bradding P, Brightling CE, Wardlaw AJ, Pavord ID. Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N Engl J Med* 2006; 354: 697-708.

249. Baluk P, Yao LC, Feng J, Romano T, Jung SS, Schreiter JL, Yan L, Shealy DJ, McDonald DM. TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *J Clin Invest* 2009; 119: 2954-2964.

250. Wenzel SE, Barnes PJ, Bleecker ER, Bousquet J, Busse W, Dahlen SE, Holgate ST, Meyers DA, Rabe KF, Antczak A, Baker J, Horvath I, Mark Z, Bernstein D, Kerwin E, Schlenker-Herceg R, Lo KH, Watt R, Barnathan ES, Chanez P, T03 Asthma Investigators. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. *Am J Respir Crit Care Med* 2009; 179: 549-558.

251. Le Cras TD, Acciani TH, Mushaben EM, Kramer EL, Pastura PA, Hardie WD, Korfhagen TR, Sivaprasad U, Ericksen M, Gibson AM, Holtzman MJ, Whitsett JA, Hershey GK. Epithelial EGF receptor signaling mediates airway hyperreactivity and remodeling in a mouse model of chronic asthma. *Am J Physiol Lung Cell Mol Physiol* 2011; 300: L414-21.

252. Heijink IH, van Oosterhout A, Kapus A. Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. *Eur Respir J* 2010; 36: 1016-1026.

253. Hamilton LM, Puddicombe SM, Dearman RJ, Kimber I, Sandstrom T, Wallin A, Howarth PH, Holgate ST, Wilson SJ, Davies DE. Altered protein tyrosine phosphorylation in asthmatic bronchial epithelium. *Eur Respir J* 2005; 25: 978-985.

254. Kung YC, Lin CC, Liaw SF, Lin MW, Chang FT. Effects of erlotinib on pulmonary function and airway remodeling after sensitization and repeated allergen challenge in Brown-Norway rats. *Respir Physiol Neurobiol* 2011; 175: 349-356.

255. Rhee CK, Kim JW, Park CK, Kim JS, Kang JY, Kim SJ, Kim SC, Kwon SS, Kim YK, Park SH, Lee SY. Effect of imatinib on airway smooth muscle thickening in a murine model of chronic asthma. *Int Arch Allergy Immunol* 2011; 155: 243-251.

256. Harvard Clinical Research Institute; National Heart, Lung, and Blood Institute (NHLBI). Effects of cKit Inhibition by Imatinib in Patients With Severe Refractory Asthma (KIA). http://clinicaltrials.gov/ct2/show/study/NCT01097694. 2013. Date last accessed: March/07 2014.

257. Humbert M, de Blay F, Garcia G, Prud'homme A, Leroyer C, Magnan A, Tunon-de-Lara JM, Pison C, Aubier M, Charpin D, Vachier I, Purohit A, Gineste P, Bader T, Moussy A, Hermine O, Chanez P. Masitinib, a c-kit/PDGF receptor tyrosine kinase inhibitor, improves disease control in severe corticosteroid-dependent asthmatics. *Allergy* 2009; 64: 1194-1201.

258. AB Science. A Phase 3 Study to Compare Efficacy and Safety of Masitinib to Placebo in Treatment of Patients with Severe Persistent Asthma Treated with Oral. http://www.clinicaltrials.gov/ct2/show/NCT01449162. 2012. Date last accessed: April 2014.

259. Yuan C, Zhou L, Cheng J, Zhang J, Teng Y, Huang M, Adcock IM, Barnes PJ, Yao X. Statins as potential therapeutic drug for asthma? *Respir Res* 2012; 13: 108-9921-13-108.

260. Zeki AA, Oldham J, Wilson M, Fortenko O, Goyal V, Last M, Last A, Patel A, Last JA, Kenyon NJ. Statin use and asthma control in patients with severe asthma. *BMJ Open* 2013; 3: 10.1136/bmjopen-2013-003314.

261. Zeki AA, Thai P, Kenyon NJ, Wu R. Differential effects of simvastatin on IL-13-induced cytokine gene expression in primary mouse tracheal epithelial cells. *Respir Res* 2012; 13: 38.

262. Ahmad T, Mabalirajan U, Sharma A, Aich J, Makhija L, Ghosh B, Agrawal A. Simvastatin improves epithelial dysfunction and airway hyperresponsiveness: from asymmetric dimethyl-arginine to asthma. *Am J Respir Cell Mol Biol* 2011; 44: 531-539.

263. Zeki AA, Bratt JM, Rabowsky M, Last JA, Kenyon NJ. Simvastatin inhibits goblet cell hyperplasia and lung arginase in a mouse model of allergic asthma: a novel treatment for airway remodeling? *Transl Res* 2010; 156: 335-349.

264. Takeda N, Kondo M, Ito S, Ito Y, Shimokata K, Kume H. Role of RhoA inactivation in reduced cell proliferation of human airway smooth muscle by simvastatin. *Am J Respir Cell Mol Biol* 2006; 35: 722-729.

265. Schaafsma D, McNeill KD, Mutawe MM, Ghavami S, Unruh H, Jacques E, Laviolette M, Chakir J, Halayko AJ. Simvastatin inhibits TGFbeta1-induced fibronectin in human airway fibroblasts. *Respir Res* 2011; 12: 113-9921-12-113.

266. Bergheim I, Luyendyk JP, Steele C, Russell GK, Guo L, Roth RA, Arteel GE. Metformin prevents endotoxin-induced liver injury after partial hepatectomy. *J Pharmacol Exp Ther* 2006; 316: 1053-1061.

267. Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, Abraham E. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2008; 295: L497-504.

268. Park CS, Bang BR, Kwon HS, Moon KA, Kim TB, Lee KY, Moon HB, Cho YS. Metformin reduces airway inflammation and remodeling via activation of AMP-activated protein kinase. *Biochem Pharmacol* 2012; 84: 1660-1670.

269. Gupta A, Sjoukes A, Richards D, Banya W, Hawrylowicz C, Bush A, Saglani S. Relationship between serum vitamin D, disease severity, and airway remodeling in children with asthma. *Am J Respir Crit Care Med* 2011; 184: 1342-1349.

270. Damera G, Fogle HW, Lim P, Goncharova EA, Zhao H, Banerjee A, Tliba O, Krymskaya VP, Panettieri RA,Jr. Vitamin D inhibits growth of human airway smooth muscle cells through growth factor-induced phosphorylation of retinoblastoma protein and checkpoint kinase 1. *Br J Pharmacol* 2009; 158: 1429-1441.

271. Song Y, Hong J, Liu D, Lin Q, Lai G. 1,25-dihydroxyvitamin D3 inhibits nuclear factor kappa B activation by stabilizing inhibitor IkappaBalpha via mRNA stability and reduced phosphorylation in passively sensitized human airway smooth muscle cells. *Scand J Immunol* 2013; 77: 109-116.

272. Leung SY, Eynott P, Nath P, Chung KF. Effects of ciclesonide and fluticasone propionate on allergen-induced airway inflammation and remodeling features. *J Allergy Clin Immunol* 2005; 115: 989-996.

273. Miller JD, Cox G, Vincic L, Lombard CM, Loomas BE, Danek CJ. A prospective feasibility study of bronchial thermoplasty in the human airway. *Chest* 2005; 127: 1999-2006.

274. Danek CJ, Lombard CM, Dungworth DL, Cox PG, Miller JD, Biggs MJ, Keast TM, Loomas BE, Wizeman WJ, Hogg JC, Leff AR. Reduction in airway hyperresponsiveness to methacholine by the application of RF energy in dogs. *J Appl Physiol (1985)* 2004; 97: 1946-1953.

275. Gordon IO, Husain AN, Charbeneau J, Krishnan JA, Hogarth DK. Endobronchial biopsy: a guide for asthma therapy selection in the era of bronchial thermoplasty. *J Asthma* 2013; 50: 634-641.

276. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, Castro M, Gaston B, Israel E, Schwartz LB, Curran-Everett D, Moore CG, Wenzel SE. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2011; 183: 299-309.

277. Stinson SE, Amrani Y, Brightling CE. D prostanoid receptor 2 (chemoattractant receptor-homologous molecule expressed on TH2 cells) protein expression in asthmatic patients and its effects on bronchial epithelial cells. *J Allergy Clin Immunol* 2015; 135: 395-406.

278. Pettipher R, Hunter MG, Perkins CM, Collins LP, Lewis T, Baillet M, Steiner J, Bell J, Payton MA. Heightened response of eosinophilic asthmatic patients to the CRTH2 antagonist OC000459. *Allergy* 2014; 69: 1223-1232.

279. Singh D, Cadden P, Hunter M, Pearce Collins L, Perkins M, Pettipher R, Townsend E, Vinall S, O'Connor B. Inhibition of the asthmatic allergen challenge response by the CRTH2 antagonist OC000459. *Eur Respir J* 2013; 41: 46-52.

280. Barnes N, Pavord I, Chuchalin A, Bell J, Hunter M, Lewis T, Parker D, Payton M, Collins LP, Pettipher R, Steiner J, Perkins CM. A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma. *Clin Exp Allergy* 2012; 42: 38-48.

281. Hall IP, Fowler AV, Gupta A, Tetzlaff K, Nivens MC, Sarno M, Finnigan HA, Bateman ED, Rand Sutherland E. Efficacy of BI 671800, an oral CRTH2 antagonist, in poorly controlled asthma as sole controller and in the presence of inhaled corticosteroid treatment. *Pulm Pharmacol Ther* 2015; 32: 37-44.

282. Miller D, Wood C, Bateman E, LaForce C, Blatchford J, Hilbert J, Gupta A, Fowler A. A randomized study of BI 671800, a CRTH2 antagonist, as add-on therapy in poorly controlled asthma. *Allergy Asthma Proc* 2017; 38: 157-164.

283. Manolis AS, Vassilikos V, Maounis TN, Chiladakis J, Cokkinos DV. Radiofrequency ablation in pediatric and adult patients: comparative results. *J Interv Card Electrophysiol* 2001; 5: 443-453.

284. Cox G, Miller JD, McWilliams A, Fitzgerald JM, Lam S. Bronchial thermoplasty for asthma. *Am J Respir Crit Care Med* 2006; 173: 965-969.

285. Pavord ID, Cox G, Thomson NC, Rubin AS, Corris PA, Niven RM, Chung KF, Laviolette M, RISA Trial Study Group. Safety and efficacy of bronchial thermoplasty in symptomatic, severe asthma. *Am J Respir Crit Care Med* 2007; 176: 1185-1191.

286. Cox G, Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, Olivenstein R, Pavord ID, McCormack D, Chaudhuri R, Miller JD, Laviolette M, AIR Trial Study Group. Asthma control during the year after bronchial thermoplasty *N Engl J Med* 2007; 356: 1327-1337.

287. Castro M, Rubin AS, Laviolette M, Fiterman J, De Andrade Lima M, Shah PL, Fiss E, Olivenstein R, Thomson NC, Niven RM, Pavord ID, Simoff M, Duhamel DR, McEvoy C, Barbers R, Ten Hacken NH, Wechsler ME, Holmes M, Phillips MJ, Erzurum S, Lunn W, Israel E, Jarjour N, Kraft M, Shargill NS, Quiring J, Berry SM, Cox G, AIR2 Trial Study Group. Effectiveness and safety of bronchial thermoplasty in the treatment of severe asthma: a multicenter, randomized, double-blind, sham-controlled clinical trial. *Am J Respir Crit Care Med* 2010; 181: 116-124.

288. Castro M, Rubin A, Laviolette M, Hanania NA, Armstrong B, Cox G, AIR2 Trial Study Group. Persistence of effectiveness of bronchial thermoplasty in patients with severe asthma. *Ann Allergy Asthma Immunol* 2011; 107: 65-70.

289. Wechsler ME, Laviolette M, Rubin AS, Fiterman J, Lapa e Silva JR, Shah PL, Fiss E, Olivenstein R, Thomson NC, Niven RM, Pavord ID, Simoff M, Hales JB, McEvoy C, Slebos DJ, Holmes M, Phillips MJ, Erzurum SC, Hanania NA, Sumino K, Kraft M, Cox G, Sterman DH, Hogarth K, Kline JN, Mansur AH, Louie BE, Leeds WM, Barbers RG, Austin JH, Shargill NS, Quiring J, Armstrong B, Castro M, Asthma Intervention Research 2 Trial Study Group. Bronchial thermoplasty: Long-term safety and effectiveness in patients with severe persistent asthma. *J Allergy Clin Immunol* 2013; 132: 1295-1302.

290. Iyer VN, Lim KG. Bronchial thermoplasty: reappraising the evidence (or lack thereof). *Chest* 2014; 146: 17-21.

291. Juniper EF, Guyatt GH, Willan A, Griffith LE. Determining a minimal important change in a disease-specific Quality of Life Questionnaire. *J Clin Epidemiol* 1994; 47: 81-87.

292. Torrego A, Sola I, Munoz AM, Roque I Figuls M, Yepes-Nunez JJ, Alonso-Coello P, Plaza V. Bronchial thermoplasty for moderate or severe persistent asthma in adults. *Cochrane Database Syst Rev* 2014; 3: CD009910.

293. Pretolani M, Dombret MC, Thabut G, Knap D, Hamidi F, Debray MP, Taille C, Chanez P, Aubier M. Reduction of airway smooth muscle mass by bronchial thermoplasty in patients with severe asthma. *Am J Respir Crit Care Med* 2014; 190: 1452-1454.

294. Jesudason EC. Airway smooth muscle: an architect of the lung? *Thorax* 2009; 64: 541-545.

295. Gordon IO, Husain AN, Charbeneau J, Krishnan JA, Hogarth DK. Endobronchial biopsy: a guide for asthma therapy selection in the era of bronchial thermoplasty. *J Asthma* 2013; 50: 634-641.

296. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J, ATS/ERS Task Force. Standardisation of spirometry. *Eur Respir J* 2005; 26: 319-338.

297. Standardized lung function testing. Official statement of the European Respiratory Society. *Eur Respir J Suppl* 1993; 16: 1-100.

298. Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax* 1997; 52: 498-501.

299. Du Rand IA, Blaikley J, Booton R, Chaudhuri N, Gupta V, Khalid S, Mandal S, Martin J, Mills J, Navani N, Rahman NM, Wrightson JM, Munavvar M, British Thoracic Society Bronchoscopy Guideline Group. British Thoracic Society guideline for

diagnostic flexible bronchoscopy in adults: accredited by NICE. *Thorax* 2013; 68 Suppl 1: i1-i44.

300. Juniper EF, Buist AS, Cox FM, Ferrie PJ, King DR. Validation of a standardized version of the Asthma Quality of Life Questionnaire. *Chest* 1999; 115: 1265-1270.

301. Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999; 14: 902-907.

302. Juniper EF, Bousquet J, Abetz L, Bateman ED, GOAL Committee. Identifying 'well-controlled' and 'not well-controlled' asthma using the Asthma Control Questionnaire. *Respir Med* 2006; 100: 616-621.

303. Jeffery P, Holgate S, Wenzel S, Endobronchial Biopsy Workshop. Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment. *Am J Respir Crit Care Med* 2003; 168: S1-17.

304. Siddiqui S, Mistry V, Doe C, Roach K, Morgan A, Wardlaw A, Pavord I, Bradding P, Brightling C. Airway hyperresponsiveness is dissociated from airway wall structural remodeling. *J Allergy Clin Immunol* 2008; 122: 335-41, 341.e1-3.

305. Cohen L, E X, Tarsi J, Ramkumar T, Horiuchi TK, Cochran R, DeMartino S, Schechtman KB, Hussain I, Holtzman MJ, Castro M, NHLBI Severe Asthma Research Program (SARP). Epithelial cell proliferation contributes to airway remodeling in severe asthma. *Am J Respir Crit Care Med* 2007; 176: 138-145.

306. Mosteller RD. Simplified calculation of body-surface area. *N Engl J Med* 1987; 317: 1098.

307. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; 33: 159-174.

308. World Health Organization. Global surveillance, prevention and control of chronic respiratory diseases: A comprehensive approach. http://www.who.int/respiratory/publications/global_surveillance/en/index.html. 2007. Date last accessed: Feb/10 2014.

309. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, Adcock IM, Bateman ED, Bel EH, Bleecker ER, Boulet LP, Brightling C, Chanez P, Dahlen SE, Djukanovic R, Frey U, Gaga M, Gibson P, Hamid Q, Jajour NN, Mauad T, Sorkness RL, Teague WG. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014; 43: 343-373.

310. O'Reilly R, Ullmann N, Irving S, Bossley CJ, Sonnappa S, Zhu J, Oates T, Banya W, Jeffery PK, Bush A, Saglani S. Increased airway smooth muscle in preschool wheezers who have asthma at school age. *J Allergy Clin Immunol* 2012.

311. James AL, Elliot JG, Jones RL, Carroll ML, Mauad T, Bai TR, Abramson MJ, McKay KO, Green FH. Airway smooth muscle hypertrophy and hyperplasia in asthma *Am J Respir Crit Care Med* 2012; 185: 1058-1064.

312. Elliot JG, Jones RL, Abramson MJ, Green FH, Mauad T, McKay KO, Bai TR, James AL. Distribution of airway smooth muscle remodelling in asthma: relation to airway inflammation. *Respirology* 2015; 20: 66-72.

313. Gupta S, Siddiqui S, Haldar P, Entwisle JJ, Mawby D, Wardlaw AJ, Bradding P, Pavord ID, Green RH, Brightling CE. Quantitative analysis of high-resolution computed tomography scans in severe asthma subphenotypes. *Thorax* 2010; 65: 775-781.

314. Gupta S, Hartley R, Singapuri A, Hargadon B, Monteiro W, Pavord ID, Sousa AR, Marshall RP, Subramanian D, Parr D, Entwisle JJ, Siddiqui S, Raj V, Brightling CE. Temporal assessment of airway remodeling in severe asthma using quantitative computed tomography. *Am J Respir Crit Care Med* 2015; 191: 107-110.

315. Hartley RA, Barker BL, Newby C, Pakkal M, Baldi S, Kajekar R, Kay R, Laurencin M, Marshall RP, Sousa AR, Parmar H, Siddiqui S, Gupta S, Brightling CE. Relationship between lung function and quantitative computed tomographic parameters of airway remodeling, air trapping, and emphysema in patients with asthma and chronic obstructive pulmonary disease: A single-center study. *J Allergy Clin Immunol* 2016; 137: 1413-1422.e12.

316. Gupta S, Hartley R, Khan UT, Singapuri A, Hargadon B, Monteiro W, Pavord ID, Sousa AR, Marshall RP, Subramanian D, Parr D, Entwisle JJ, Siddiqui S, Raj V, Brightling CE. Quantitative computed tomography-derived clusters: redefining airway remodeling in asthmatic patients. *J Allergy Clin Immunol* 2014; 133: 729-38.e18.

317. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma *N Engl J Med* 2002; 346: 1699-1705.

318. Fox SB, Leek RD, Weekes MP, Whitehouse RM, Gatter KC, Harris AL. Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. *J Pathol* 1995; 177: 275-283.

319. Hansen S, Grabau DA, Rose C, Bak M, Sorensen FB. Angiogenesis in breast cancer: a comparative study of the observer variability of methods for determining microvessel density. *Lab Invest* 1998; 78: 1563-1573.

320. Carroll NG, Cooke C, James AL. Bronchial blood vessel dimensions in asthma. *Am J Respir Crit Care Med* 1997; 155: 689-695.

321. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches *Nat Med* 2012; 18: 716-725.

322. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial *Lancet* 2002; 360: 1715-1721.

323. Castro M, Wenzel SE, Bleecker ER, Pizzichini E, Kuna P, Busse WW, Gossage DL, Ward CK, Wu Y, Wang B, Khatry DB, van der Merwe R, Kolbeck R, Molfino NA, Raible DG. Benralizumab, an anti-interleukin 5 receptor alpha monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. *Lancet Respir Med* 2014; 2: 879-890.

324. Xue L, Gyles SL, Wettey FR, Gazi L, Townsend E, Hunter MG, Pettipher R. Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. *J Immunol* 2005; 175: 6531-6536.

325. Xue L, Barrow A, Pettipher R. Novel function of CRTH2 in preventing apoptosis of human Th2 cells through activation of the phosphatidylinositol 3-kinase pathway. *J Immunol* 2009; 182: 7580-7586.

326. Xue L, Salimi M, Panse I, Mjosberg JM, McKenzie AN, Spits H, Klenerman P, Ogg G. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol* 2014; 133: 1184-1194.

327. Gervais FG, Cruz RP, Chateauneuf A, Gale S, Sawyer N, Nantel F, Metters KM, O'neill GP. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. *J Allergy Clin Immunol* 2001; 108: 982-988.

328. Monneret G, Gravel S, Diamond M, Rokach J, Powell WS. Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood* 2001; 98: 1942-1948.

329. Juniper EF, Frith PA, Dunnett C, Cockcroft DW, Hargreave FE. Reproducibility and comparison of responses to inhaled histamine and methacholine. *Thorax* 1978; 33: 705-710.

330. Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, Casaburi R, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Hankinson J, Jensen R, Johnson D, Macintyre N, McKay R, Miller MR, Navajas D, Pellegrino R, Viegi G. Standardisation of the measurement of lung volumes. *Eur Respir J* 2005; 26: 511-522.

331. Macintyre N, Crapo RO, Viegi G, Johnson DC, van der Grinten CP, Brusasco V, Burgos F, Casaburi R, Coates A, Enright P, Gustafsson P, Hankinson J, Jensen R, McKay R, Miller MR, Navajas D, Pedersen OF, Pellegrino R, Wanger J. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J* 2005; 26: 720-735.

332. American Thoracic Society, European Respiratory Society. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005; 171: 912-930.

333. Britten KM, Howarth PH, Roche WR. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. *Biotech Histochem* 1993; 68: 271-280.

334. Busse WW, Wenzel SE, Meltzer EO, Kerwin EM, Liu MC, Zhang N, Chon Y, Budelsky AL, Lin J, Lin SL. Safety and efficacy of the prostaglandin D2 receptor antagonist AMG 853 in asthmatic patients. *J Allergy Clin Immunol* 2013; 131: 339-345.

335. Kostenis E, Ulven T. Emerging roles of DP and CRTH2 in allergic inflammation. *Trends Mol Med* 2006; 12: 148-158.

336. Dyrda P, Tazzeo T, DoHarris L, Nilius B, Roman HN, Lauzon AM, Aziz T, Lukic D, Janssen LJ. Acute response of airway muscle to extreme temperature includes disruption of actin-myosin interaction. *Am J Respir Cell Mol Biol* 2011; 44: 213-221.

337. Lackie PM, Baker JE, Gunthert U, Holgate ST. Expression of CD44 isoforms is increased in the airway epithelium of asthmatic subjects. *Am J Respir Cell Mol Biol* 1997; 16: 14-22.

338. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999; 104: 123-133.

339. Saunders R, Biddle M, Wright A, Chachi L, Sutcliffe A, Berair R, Brightling C. DP2/CRTh2 is expressed by ASM cells in asthma and its inhibition suppresses ASM migration. *Eur Respir J* 2016; 48: PA4653.