University of Southampton

Faculty of Medicine

Activation of the Epithelial Mesenchymal Trophic Unit (EMTU) and Markers of Remodelling Associated with Asthma Persistence and Remission in Early Adulthood

Volume 1 of 1

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Submitted for DM

February 2015

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Clinical and Experimental Sciences

Doctor of Medicine

ACTIVATION OF THE EPITHELIAL MESENCHYMAL TROPHIC UNIT (EMTU) AND MARKERS OF REMODELLING ASSOCIATED WITH ASTHMA PERSISTENCE AND REMISSION IN EARLY ADULTHOOD

By Dr Sian Louise Evans

Asthma is common childhood illness that sometimes persists into adulthood but remission from childhood asthma is common in the second decade of life. However, even in young adults with remission of asthma symptoms, there often persisting bronchial hyper-responsiveness and airway inflammation and the pathophysiological characteristics of asthma in remission remain unclear.

This thesis compares markers of remodelling, inflammation and epithelial repair in young adults recruited from the well-established 1989 Isle of Wight Birth Cohort. They formed three groups: those with persistent asthma, asthma remission and never asthma. They underwent clinical characterisation, bronchial provocation testing with mannitol and fibreoptic bronchoscopy to obtain bronchial biopsy samples.

The samples obtained were analysed for cells involved in the inflammatory response, markers of remodelling and markers of epithelial damage and repair. This allowed us to determine the similarities and differences between those young adults who had grown out of their childhood asthma and those who had not.

List of Contents

List of Figures Page 13

List of Tables Page 15

Declaration of Authorship Page 17

Acknowledgements Page 19

Abbreviations Page 21

CHAPTER 1 – INTRODUCTION

1.1 ATOPY, ALLERGIC DISASE AND ASTHMA Page 26

1.1.1 Allergen Avoidance

1.1.2 Immunotherapy

1.1.3 Asthma Initiation

1.1.4 Asthma Transition during Adolescence

1.1.5 Gender Differences in Asthma

1.2 INFLAMMATION Page 43

1.2.1 The Inflammatory Cascade

1.2.1.1. Acute and late-phase response

1.2.1.2. Chronic inflammation

1.2.2 Inflammatory cells in asthma

1.2.2.1 Eosinophils

1.2.2.2. Mast cells

1.2.2.3 Neutrophils

1.2.3 Therapeutics and the Inflammatory Cascade

1.2.3.1 Corticosteroids and long-acting β_2-adrenoceptor agonists

1.2.3.2 Anti-IgE

1.2.3.3 IL-5

1.2.4 New Targets for Therapeutics

1.2.4.1 IL-5

1.2.4.2 IL-4 AND IL-13

1.2.4.3 IL-17

1.3 THE EPITHELIAL MESENCHYMAL TROPHIC UNIT (EMTU) Page 53

1.3.1 Epithelial Function

1.3.2 The Asthmatic Epithelium

1.3.2.1 Abnormal repair mechanisms: Epidermal Growth Factor Receptor (EGFR) and $p21^{waf}$

1.3.3 Epithelial-Mesenchymal Communication

1.3.3 The Role of Epithelium-derived Cytokines in Asthma

1.3.3.1 IL-33

1.3.3.2 IL-8

1.4 AIRWAY REMODELLING AND BRONCHIAL HYPER-RESPONSIVENESS Page 61

1.4.1 Epithelial Abnormalities in Remodelling

1.4.2 Subepithelial Fibrosis

1.4.2.1 Lamina reticularis fibrosis

1.4.2.2 Submucosal extracellular matrix

1.4.2.3 Deposition and degradation of extracellular matrix proteins

1.4.3 Airway Smooth Muscle

1.4.4 Angiogenesis

1.4.5 Bronchial Hyper-responsiveness and Remodelling

1.4.6 Remodelling and the EMTU

1.4.7 Other Factors involved in Remodelling

1.4.7.1 Transforming growth factor-β (TGF-β)

1.4.7.2 Periostin

1.4.8 Viral Infection and Remodelling

1.5 THE ISLE OF WIGHT 1989 BIRTH COHORT Page 75

CHAPTER 2 – MATERIALS, METHODS AND ANALYSIS

2.1 THE STUDY Page 77

2.1.1 The Hypothesis

2.1.2 The Aims of the Study

2.1.3 Ethics and Research and Development

2.1.4 Power Calculation

2.1.6 Study Personnel and Recruitment

2.1.5 Exclusion Criteria

2.2 CLINICAL ASSESSMENT Page 83

2.2.1 Visit One

2.2.2 Questionnaires

2.2.3 Skin Prick Testing

2.2.4 Spirometry and BHR Assessment

2.2.5 Urine Testing

2.2.6 Visit two

2.2.7 Blood Sampling

2.2.8 Reversibility

2.2.9 Bronchoscopy

2.3 ADVERSE EVENTS Page 93

2.4 IMMUNOHISTOCHEMISTRY Page 95

2.4.1 Biopsy Preparation

2.4.2 General Methods

2.4.3 Neutrophils, Eosinophils and Mast cells

2.5 QUANTITATION OF IMMUNOHISTOCHEMICAL STAINING Page 97

2.5.1 Collagen I and III and Collagen Band Thickness (lamina reticularis)

2.5.2 α-SMA Volume

2.5.3 Periostin in Subepithelium

2.5.4 EGFR, $p21^{waf}$, Il-8 and in TGF-β_2 in Epithelium

2.5.5 IL-33 in Epithelium

2.5.6 Neutrophils, Eosinophils and Mast Cells – Cell Counting

CHAPTER 3 CLINICAL CHARACTERISATION

3.1 DEFINITIONS Page 99

3.2 PARTICIPANTS SCREENED Page 101

3.3 GENDER AND ANTHROPOMETRIC MEASUREMENTS Page 103

3.3.1 Height

3.3.2 Weight

3.3.3 Body Mass Index

3.4 SOCIOECONOMIC INDICES Page 106

3.4.1 Employment and Education Status

3.4.2 Tenure of Housing

3.4.3 Family Income

3.4.4 Smoking Status

3.4.5 Leisure Activities

3.5 EXPOSURE TO ALLERGENS Page 110

3.5.1 Air and Traffic Pollution

3.5.2 Pets

3.5.3 Moulds and Fungus

3.6 ATOPY Page 112

3.6.1 Atopic Status and Skin Prick Testing

3.6.2 Co-morbidity with other Allergic Disease

3.7 FEV_1 AND AIRWAY OBSTRUCTION Page 115

3.7.1 Percentage predicted FEV_1

3.7.2 Percentage predicted FEV_1

3.8 USE OF PARACETAMOL AND IBUPROFEN Page 117

3.9 CHARACTERISATION OF ASTHMATIC PARTICIPANTS Page 119

3.9.1 Use of Asthma Medication and Asthma Severity

3.9.2 Asthma Symptoms and Impact on Quality of Life

3.10 CHARACTERISATION OF REMISSION PARTICIPANTS Page 120

CHAPTER 4 BRONCHIAL HYPER-RESPONSIVENESS (BHR), AIRFLOW REVERSIBILITY AND MARKERS OF INFLAMMATION

4.1 ASSESSMENT OF BHR (MANNITOL CHALLENGE) Page 123

4.2 AIRFLOW REVERSIBILITY Page 125

4.3 MARKERS OF INFLAMMATION Page 127

4.3.1 Mast Cells

4.3.2 Eosinophils

4.3.3 Neutrophils

CHAPTER 5 MARKERS OF REMODELLING

5.1 COLLAGEN BAND THICKNESS Page 131

5.2 SUBEPITHELIAL COLLAGEN I DEPOSITION Page 131

5.3 SUBEPITHELIAL COLLAGEN III DEPOSITION Page 131

5.4 SUBEPITHELIAL PERIOSTIN Page 132

5.5 ALPHA SMOOTH MUSCLE ACTIN (α SMA) Page 132

CHAPTER 6 MARKERS OF EPITHELIAL DAMAGE

6.1 ANALYSIS OF EGFR Page 143

6.2 ANALYSIS OF $P21^{WAF}$ Page 143

6.3 ANALYSIS OF IL-8 Page 143

6.4 ANALYSIS OF IL-33 Page 144

6.5 ANALYSIS OF TGF-β_2 Page 144

CHAPTER 8 DISCUSSION AND CONCLUSION

Page 157

8.1 STRENGTHS AND LIMITATIONS Page 165

8.2 CONCLUSION Page 167

APPENDICES AND ACCOMPANYING MATERIALS

APPENDIX I Study Personnel

APPENDIX II Accompanying Materials Used

APPENDIX III Markers of Remodelling the Wessex Severe Asthma cohort: a pre-study project

LIST OF REFERENCES

List of Figures

Figure 1: Determinants influencing the development of allergic diseases

Figure 2a: Schematic representation of how epithelial injury and aberrant repair interact with inflammatory components in chronic asthma.

Figure 2b: Immunofluorescent confocal microscopy of whole mounts of bronchial biopsies from a normal *(left)* and asthmatic *(right)* airway. The section has been stained with an antibody that identifies the TJ protein ZO-1 *(green)*, with the epithelial cell nuclei stained *red*. Note the lack of TJ organization in the asthmatic epithelium when viewed in the horizontal and vertical planes.

Figure 3: Schematic representation of the sentinel role of the airway epithelium in coordinating mucous metaplasia and chronic airway inflammation.

Figure 4: Schematic representation of the interrelationships between epithelial injury and aberrant repair and chronic inflammation in moderate-severe asthma. Immune and inflammatory cells interact with structural cells both to maintain the inflammatory response and to drive airway wall remodelling.

Figure 5: Mechanisms via which direct and indirect stimuli induce airflow limitation.

Figure 6: Consort diagram to demonstrate recruitment into the study

Figure 7a: Mean height (m) and weight (kg) stratified by sex in each asthma group.

Figure 7b. Body mass index (kg/m^2) by asthma group

Figure 8. Atopic status stratified by asthma group

Figure 9. Percentage FEV_1 compared between asthma groups pre- and post-bronchodilator with significance values. Bars indicate 95% Confidence intervals

Figure 10: Dot plot demonstrating mean FEV_1 reversibility (%) with significance values (Horizontal line represents mean)

Figure 11: Dot plot of mast cells/mm^2 (horizontal line represents median)

Figure 12: Dot plot of eosinophils/mm^2 (horizontal line represents median)

Figure 13: Dot plot of neutrophils/mm^2 (horizontal line represents median)

Figure 14: Asthma group of samples analysed in the Wessex Severe Asthma Cohort

Figure 15: Boxplot of collagen band thickness (µm) in asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

Figure 16: Boxplot of collagen band thickness (µm) between asthma severity groups compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

Figure 17: Boxplot of subepithelial collagen I deposition (%) in asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

Figure 18: Boxplot of subepithelial collagen I deposition (%) between asthma severity groups compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

Figure 19: Boxplot of subepithelial collagen III deposition (%) in asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

Figure 20: Boxplot of subepithelial collagen III deposition (%) in asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

List of Tables

Table 1a Power calculations based on non-parametric Wilcoxon rank sum test.

Table 1b Power calculations based on two sample t-tests.

Table 2a: Medications to be stopped (and timescale) before performing the bronchial provocation test.

Table 2b: BTS asthma severity staging

Table 3: Incremental mannitol dosing regime

Table 4: Adverse events

Table 5: Characteristics of participants entering the study

Table 6: Exposure to allergens and pollutants

Table 7: Use of paracetamol and ibuprofen by asthma group

Table 8: Characteristics of asthma participants

Table 9: Result of mannitol challenge test by asthma group

Table 10: Mean FEV_1 reversibility (%) with significance values

Table 11: Details of primary and secondary antibodies

Table 12: Median mast cells/mm^2 with IQR and significance testing

Table 13: Median eosinophils/mm^2 with IQR and significance testing

Table 14: Median neutrophils/mm^2 with IQR and significance testing

Table 15: Collagen band thickness

Table 16: Subepithelial collagen I deposition

Table 17: Subepithelial collagen III deposition

Table 18: Subepithelial periostin deposition

Table 19: Alpha smooth muscle actin (α SMA)

Table 20: BM thickness as reported by other studies

Table 21: Epithelial deposition of EGFR

Table 22: Epithelial deposition of $p21^{waf}$

Table 23: Epithelial deposition of IL-8

Table 24: Epithelial deposition of IL-33

Table 25: Epithelial deposition of $TGF\beta_2$

Table 26: Mean collagen band thickness (µm) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohort

Table 27: Mean subepithelial collagen I deposition (%) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohort

Table 28: Mean subepithelial collagen III deposition (%) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohor

DECLARATION OF AUTHORSHIP

I, Sian Louise Evans, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself (please see page 74 section 2.1.4)
7. Parts of this work have been published as:

2013 Sept	Markers of airway remodelling in young adults with clinical remission of asthma; Evans S. ERS Barcelona, poster presentation
2013 Sept	Periostin as a marker of remodelling in young adults with clinical remission of asthma; Evans S. ERS Barcelona, poster presentation

Signed: .....

Date:.....

Acknowledgements

I would like to express my gratitude to my thesis supervisors Professor Hasan Arshad. Professor Donna Davies and Professor Peter Howarth who have been mentors to me throughout the writing of this thesis.

Also many thanks to the David Hide Asthma and Allergy Centre and the staff there; all of whom supported me throughout the duration of the study beyond the call of duty. Thank you to Hongmei Zhang who assisted me with the power calculation that was completely beyond me.

Many thanks to the Southampton NIHR Wellcome Trust Clinical Research Facility and the nurses Sandra Pink, Laura Presland, Shuna Egerton, Caroline Smith, Kerry Thorpe and Lorraine Hewitt who assisted with the bronchoscopies and administrative component.

To the Histochemistry Research Unit at University Hospital Southampton and its staff including Susan Wilson, John Ward, Helen Rigden and Jenny Norman; a big thank you for your endless patience with my struggles to master immunohistochemistry, and thank you to Laurie Lau who has supported and guided generations of Southampton researchers through their studies.

And finally a special mention to two colleagues, who are now good friends, Stephen Potter and Frances Mitchell. Thank you Stephen for your sense of humour that kept me sane, for your brilliant IT skills, for hunting down my purple folder all over the Island (including the bus depot) and much more. Thank you Frances for learning to be a bronchoscopy nurse and travelling to Southampton at considerable inconvenience to yourself on those cold dark mornings. Thank you for becoming the best bronchoscopy nurse that I have worked with and putting such love and care into the study.

Abbreviations

αSMA	Alpha smooth muscle actin
AEC	3-amino 9-ethylcarbazole
AMP	Adenosine monphosphate
AR	Asthma remission
ASM	Airway smooth muscle
ASMC	Airway smooth muscle cells
BAL	Bronchoalveolar lavage
BEC	Bronchial epithelial cells
BHR	Bronchial hyper-responsiveness
BMI	Body mass index
BPT	Bronchial provocation testing
BTS	British Thoracic Society
CBT	Collagen band thickness
ClinR	Clinical remission (of asthma)
COPD	Chronic obstructive pulmonary disease
CoR	Complete remission (of asthma)
CRF	Case Report form
DAB	Diaminobenzidine
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptors
EM	Electron microscopy
EMTU	Epithelial mesenchymal trophic unit
FEV_1	Forced expiratory volume in 1 second
FENO	Fractional exhaled nitric oxide
FGF	Fibroblast growth factor

FVC	Forced vital capacity
GMA	Glycol methacrylate
GM-CSF	Granulocyte macrophage colony stimulating factor
GP	General Practitioner
H_2O_2	Hydrogen peroxide
ASMC	Airway smooth muscle cells
HCG	Human chorionic gonadotrophin
HDM	House dust mite
ICAM	Intracellular adhesion molecule
ICS	Inhaled corticosteroids
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
INNOVATE	The INvestigation of Omalizumab in seVere Asthma TrEatment
IPF	Idiopathic pulmonary fibrosis
ISAAC	International Study of Asthma and Allergies in Childhood
IW	Intermittent wheezer
LABA	Long-acting β_2 agonist
MAAS	Manchester Asthma and Allergy Study
mAb	Monoclonal antibody
MBP	Major basic protein
MHC	Major histocompatibility complex
MMP	Matric metallopteinases
NA	Never Asthma
NHS	National Health Service
NKT	Natural killer T (cells)
NOE	Neutrophil elastase
PA	Persistent asthma
PAR	Protease activated receptors
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factors
PEFR	Peak expiratory flow rate

PIS	Patient Information Sheet
RANTES	Regulated on activation, normal T cell expressed and secreted
RBM	Reticular basement membrane
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RV	Rhinovirus
SABA	Short-acting β_2 agonist
SCIT	Subcutaneous immunotherapy
SD	Standard deviation
SIGN	Scottish Intercollegiate Guidelines Network
SLIT	Sublingual immunotherapy
SOP	Standardized operating policy
SPT	Skin prick testing
STAT6	Signal transducer and activator of transcription 6
TER	Transepithelial resistance
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinases
TH	T Helper
TJ	Tight junctions
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRAILS	TRacking Adolescents' Individual Lives SurveY
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor

CHAPTER 1 - INTRODUCTION

Asthma has been recognised since the time of the ancient Greeks as it is thought to be derived from 'ἄσθμα' the Greek word for 'panting'. In the 17th century Osler wrote in '*Principles and Practice of Medicine'* about the asthma syndrome in detail; defining features astonishingly close to those we use to define the disease to this day (Girard 1981). He described a familial syndrome associated with hayfever usually starting in childhood and sometimes persisting into old age. He also described pathological features of swelling of the bronchial mucous membrane and small bronchiole inflammation and clinical features of bronchial muscle spasm, gelatinous sputum and 'paroxysms' exacerbated by pollutants, high emotion, diet and infection. Today asthma is still defined both clinically and pathologically. It is usually described as a clinical syndrome of variable airflow limitation and recurring symptoms of chest tightness, shortness of breath, cough and wheeze. It can be atopic or non-atopic and is associated with bronchial hyper-responsiveness (BHR) and airway inflammation (Tattersfield, Knox et al. 2002).

Asthma is one of the most common chronic conditions in the Western world affecting 1 in 7 children and 1 in 12 adults, in total 5.1 million people in the UK. Asthma prevalence has been increasing by 5% every decade, although in areas with higher prevalence this seems to have stabilised in recent years (Asher, Montefort et al. 2006). It is responsible for 1500 avoidable deaths and 20 million lost working days per year; the annual UK cost to NHS of asthma is estimated at about £2.5 billion. Current asthma therapy does not offer either prevention or cure, nor does it alter the natural history of the disease.

1.1 ATOPY, ALLERGIC DISEASE AND ASTHMA

Atopy is a genetically determined predisposition to generate specific IgE against common allergens in a type 1 hypersensitivity reaction. The specific antibodies can be detected in the blood or skin and the individual is considered sensitised' to this allergen (Burrows, Martinez et al. 1989). Clinically, atopy is defined as presence of specific antibodies in serum or a positive reaction to one or more allergens on skin test. A sensitised individual can go on to develop an allergic disease (such as asthma, eczema, rhinitis and food allergy) when they are exposed to the allergen at a later time. At the present time atopy affects over 40% of the developed world and yet only a relatively small proportion develop clinically significant allergic disease (Beasley, Keil et al. 1998). This suggests that there must be environmental factors that allow the atopic tendency to express itself as a clinical entity.

Allergic disease is a significant cause of morbidity in childhood affecting 25% of children in Western countries (Asher, Montefort et al. 2007, Torres-Borrego, Molina-Teran et al. 2008). Co-morbidity between allergic diseases has mainly been studied in high-risk cohorts where it has been shown to be substantial and associated with both disease severity and persistence (Leynaert, Neukirch et al. 2004, Bousquet, Khaltaev et al. 2008, Bertelsen, Carlsen et al. 2010).

Asthma is a relapsing and remitting clinical syndrome comprising variable airflow obstruction which leads to the symptoms of wheeze, cough and shortness of breath. This disease often starts in childhood and its sufferers have a high incidence of atopy, however, not all forms of asthma demonstrate atopic sensitisation and there are characteristic differences between atopic and non-atopic states, particularly that those with atopic asthma often have a more severe form if the disease and are less likely to achieve remission (Kurukulaaratchy, Fenn et al. 2004, Kurukulaaratchy, Matthews et al. 2005, Chawes, Kreiner-Moller et al. 2009, Keil, Bockelbrink et al. 2010).

Atopy and asthma are both increasing in prevalence in the Western World. The biggest growth in atopic disease appears to have occurred between 1960 and 1990 (Beasley, Keil et al. 1998) in particular in Westernised countries, whereas asthma and hayfever are virtually unknown in rural areas of Africa and Eastern Russia (Woolcock and Peat 1997).

The increase cannot be explained by genetic factors alone and therefore environmental influences are thought to be responsible and many theories have been proposed to explain this phenomenon.

Ambient air pollution can undoubtedly lead to exacerbation of *pre-existing* respiratory diseases such as asthma, however there are several studies that demonstrate lower levels of atopy, hayfever, asthma and wheezing in heavily polluted areas compared with less-polluted areas (Vonmutius, Fritzsch et al. 1992, Braback, Breborowicz et al. 1994, Vonmutius, Martinez et al. 1994, Braback, Breborowicz et al. 1995, Nowak, Heinrich et al. 1996). Therefore the role of air pollution in the development of atopy and allergic disease remains controversial. Similarly while traffic exposure increases the amount of respiratory symptoms (Wjst, Reitmeir et al. 1993) (Ishizaki and Koizumi 1985) (Hirsch, Weiland et al. 1999) it seems to have little impact on the prevalence of atopic disease per se (Oosterlee, Drijver et al. 1996, Nakai, Nitta et al. 1999).

The 'hygiene' hypothesis associates increasing levels of atopy in the developed world with lack of exposure to previously prevalent bacterial, viral and parasitic infection 36; to support this, developing countries often have higher total levels of IgE to parasitic infection but lower levels of specific IgE to common allergens (Larrick, Buckley et al. 1983)16 36. Also children who go to pre-school day-care and mix with other children (thus presumably are exposed to higher levels of childhood infection) have lower levels of allergies later in life (Kramer, Heinrich et al. 1999). Interestingly, several studies have demonstrated that children who live on farms have significantly lower levels of atopy than their peers from the same rural areas who do not live on farms; and a modest reduction in allergic disease (Braun-Fahrlander, Gassner et al. 1999, Riedler, Eder et al. 2000, Von Ehrenstein, Von Mutius et al. 2000). There may be many confounding factors to explain this but investigators did not think them of sufficient importance to explain the strength of the inverse correlation. Again, early life exposure to allergens and a diverse microbiome (close contact with animals, unpasteurised milk) have been suggested as possible explanations (Ring, Kramer et al. 2001).

Socioeconomic indices have been linked to atopic status and it has been noted that atopy increases with higher educational status of parents (Heinrich, Popescu et al. 1998) and

higher socioeconomic status (Williams, Strachan et al. 1994, Strachan, Harkins et al. 1997). This is difficult to interpret as other factors are likely to contribute such as access to healthcare, awareness of disease, nutrition and breastfeeding. Certainly higher numbers of siblings has been shown to be inversely related to the prevalence of hayfever, atopic eczema and atopy (Golding and Peters 1987, Strachan 1989, Strachan, Taylor et al. 1996, Jarvis, Chinn et al. 1997, Matricardi, Rosmini et al. 1997, Olesen, Ellingsen et al. 1997, Rasanen, Laitinen et al. 1997, Strachan, Harkins et al. 1997, Matricardi, Franzinelli et al. 1998). In particular the younger siblings appear to be most protected; this does not seem to be related to factors such as age (Strachan, Taylor et al. 1996). A theory has been proposed in a similar vein to the 'hygiene hypothesis' whereby 'unhygienic' contact with older siblings in early life has a protective role against allergic disease later in life (Strachan 1989).

Higher levels of gastrointestinal infections in young children (Bjorksten, Naaber et al. 1999) and adults (Matricardi, Franzinelli et al. 1998) have also been shown to lead to lower rates of allergy which may suggest that diverse gut microbiology may have a negative effect on developing allergy. Lower respiratory infection with RSV is associated with increased wheeze later in childhood (Stein, Sherrill et al. 1999) but this is not related to atopy and in fact this increased risk of wheeze has been shown only to last until early adolescence and does not develop into persistent asthma (Sims, Downham et al. 1978, Pullan and Hey 1982, Long, McBride et al. 1995). Several population studies (India, Fiji, and Eastern Europe) have demonstrated an inverse relationship between bacterial lower respiratory infections and asthma (Anderson 1974, Flynn 1994, Flynn 1994, Vonmutius, Martinez et al. 1994, Nowak, Heinrich et al. 1996). It has been postulated that exposure to bacterial infection drives the immune system away from atopic expression and allergic disease.

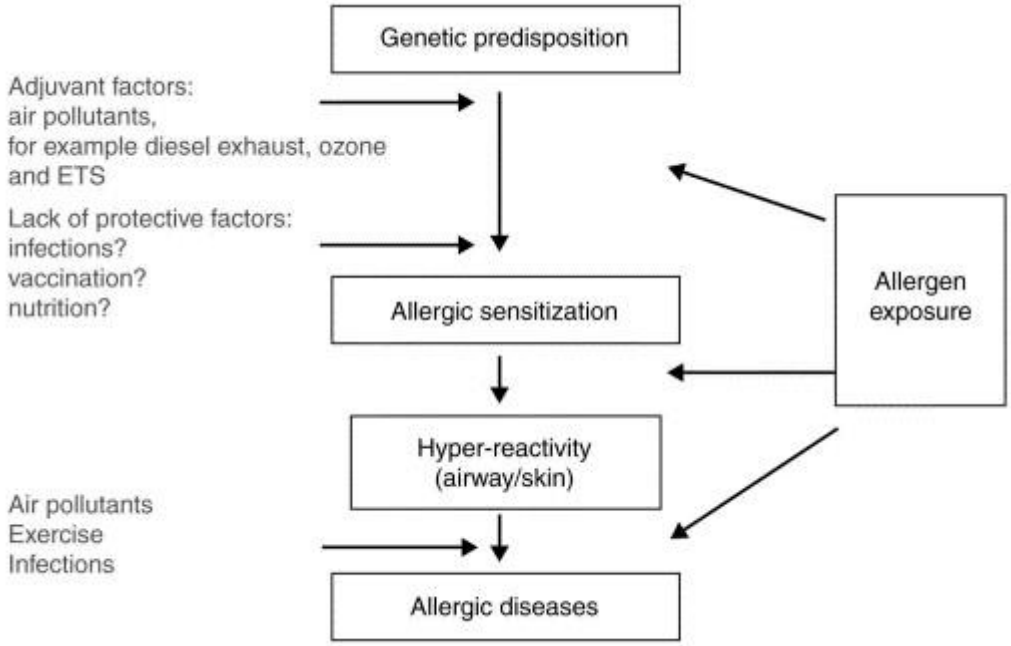
The role of allergens in the development of allergic disease has been the subject of much interest and there is conflicting evidence regarding allergen exposure. Many studies have shown evidence linking increased allergen exposure to increased sensitization in children (Lau, Falkenhorst et al. 1989, Kuehr, Frischer et al. 1994, Wahn, Lau et al. 1997). However the correlation between sensitisation (to HDM) and asthma is less clear cut, *Sporik et al* demonstrated a strong correlation between wheezing and sensitisation to HDM (Sporik, Holgate et al. 1990) but several studies looking at mite-free environments (the Alps, Tuscon,

inland Australia) have not detected a reduced prevalence of asthma as might be expected (Charpin, Birnbaum et al. 1991, Peat and Woolcock 1991, Sporik, Ingram et al. 1995, Halonen, Stern et al. 1997). It would appear that the timing and level of exposure to allergens combined with the genetic susceptibility of an individual determines the outcome of in terms of the manifestation of allergic disease.

It is likely that a complex interplay of factors have contributed to the rise of atopy and allergic diseases in the last 60 years that cannot easily be explained by one single theory, rather a combination of several. Our 'modern society' in which there is increased exposure to HDM within the home, smaller families, better hygiene and decreased exposure to infection is likely to have influenced this increase (see figure 1).

From a clinical standpoint much interest has been expressed in targeting allergen exposure in asthma. This has led to the development of two different therapies at either end of the spectrum: avoidance strategies and 'desensitization' i.e. allergen immunotherapy.

Figure 1 Determinants influencing the development of allergic diseases (Ring, Kramer et al. 2001)



1.1.1 Allergen Avoidance

Exposure to an allergen and a subsequent response in the form of asthma symptoms are often obvious to both patient and clinician; one of the most common triggers for asthma attacks in sensitised individuals is house dust mite. House dust is a mixture containing many different allergens, but the major allergen is derived from house dust mite (HDM), especially the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. A common site for house dust mites is the bed, where pillows, quilts and mattresses often serve as reservoirs for the allergen. Carpets and upholstered furniture may also contain high mite levels (1988, Tovey 1992). There has been much interest and discussion about allergen avoidance as both primary and secondary prophylaxis. Primary prophylaxis is an intervention introduced before the onset of disease and designed to reduce incidence. Secondary prophylaxis is an intervention introduced after disease onset to reduce/control symptoms.

Primary prevention

Early life exposure to HDM increases the risk of becoming sensitised to HDM (Wahn, Lau et al. 1997) and this in turn appears to be a risk factor for the development of asthma. A few studies have suggested that high early life exposure to HDM increases the risk of subsequent childhood asthma (Arshad, Bateman et al. 2003), however one UK study showed that low levels of HDM in early life *increased* the risk of respiratory symptoms at 5 years (Cullinan, MacNeill et al. 2004). These are observational studies, however, and there appears to be considerable interactions with other factors such as birth order and family history.

Epidemiological studies have suggested that close contact with a domestic animal in early life may reduce later prevalence of asthma and allergy (Platts-Mills, Vaughan et al. 2001). This has raised the question of whether high-dose allergen exposure could lead to an immune 'tolerance.'

Intervention studies attempting to reduce exposure to HDM have been inconsistent. Avoidance is difficult and time consuming; participants are required to use hypoallergenic bedding covers, avoid carpeted floors and soft toys, frequently vacuum soft furnishings and/or use acaricide sprays. Most studies have attempted to target children at 'high risk' of asthma. The Study of Primary Prevention of Allergy in Europe assessed the effect of simple HDM avoidance methods and demonstrated a decrease in HDM sensitisation at 1 year (Halmerbauer, Gartner et al. 2003) but not at 2 years and no reduction in the clinical manifestation of allergic disease (Tsitoura, Nestoridou et al. 2002, Horak, Matthews et al. 2004). The Prevention and Incidence of Asthma and Mite Allergy Study had similar findings also demonstrating reduced sensitisation but no difference in allergic disease manifestation up to the age of 8 years old (Brunekreef, Smit et al. 2002, Gehring, De Jongste et al. 2012). The Manchester Asthma and Allergy Study (MAAS) used a more intensive regime of HDM avoidance during infancy and pregnancy; they demonstrated a significant decrease in exposure to HDM, cat and dog allergens but no reduction in sensitisation (Custovic, Simpson et al. 2000). At 1 year there was no difference in mild wheeze or cough but a significant reduction in the intervention group in episodes of severe wheezing (Custovic, Simpson et al. 2001). A primary prevention study carried out on the Isle of Wight

focussing on a multifactorial avoidance technique during infancy demonstrated a reduction in sensitisation to most allergens up to 8 years of age with reduction in the prevalence of asthma, atopic dermatitis and asthma at 1 year (Arshad and Hide 1992) and 8 years (Arshad, Bateman et al. 2003). At 2 years (Hide, Matthews et al. 1994) and 4 years (Hide, Matthews et al. 1996) only the reduction in atopic dermatitis was significant. A Canadian study showed a reduction in doctor-diagnosed asthma at 5 years in the intervention group but no difference in other allergic disease, skin prick testing or bronchial hyper-responsiveness (Chan-Yeung, Ferguson et al. 2005). Other studies have demonstrated increased allergy in later life but paradoxically better lung function in the intervention group (Woodcock, Lowe et al. 2004).

A Cochrane review summarised in the British Thoracic Society (BTS)/Scottish Intercollegiate Guidelines Network (SIGN) Asthma Guidelines considers studies using a 'multifaceted approach' which involves manipulating dietary allergens and environmental exposures and demonstrates a reduction by half in doctor diagnosed asthma in later childhood (Maas, Dompeling et al. 2011). This is not consistently associated with a reduction in respiratory symptoms however. Therefore the BTS/SIGN guidelines conclude that a multifaceted approach may be considered in children at high risk of asthma if the family are motivated and prepared to go to considerable inconvenience (2014).

Secondary prevention

In individuals with asthma, increased allergen exposure is associated with increased asthma symptoms, BHR and deterioration in lung function (Peat, Salome et al. 1990, Sporik, Holgate et al. 1990, Plattsmills, Thomas et al. 1992). However it has yet to be demonstrated convincingly that allergen avoidance can reduce morbidity. Studies have mostly been small and of differing and inconsistent methodology. Mattress and pillow barrier systems have had some success. *Halken et al* demonstrated a significantly reduced requirement for inhaled corticosteroids in children in her intervention group (Halken, Host et al. 2003) and in the study by *Van den Bemt* an increase in morning peak flow was seen but this did not translate into an improvement in medication use or symptoms (van den Bemt, van Knapen et al. 2004). A prospective randomised, double blind placebo controlled study of 126 British adults in 2007 compared HDM avoidance with self-management vs self-management alone

and found no benefit in the use of inhaled corticosteroids (ICS), peak flow or asthma symptoms (de Vries, van den Bemt et al. 2007). A further double blind placebo controlled randomised study in adults also demonstrated no benefit from using allergen-impermeable covers alone (Woodcock, Forster et al. 2003). A Cochrane review on house dust mite control measures was unable to recommend this as a cost-effective measure for improving asthma symptoms (Gotzsche and Johansen 2008). A clinical review of 54 trials in 2008 failed to show any statistically significant benefit associated with these measures (Gotzsche and Johansen 2008).

There is limited and conflicting information as to the possible benefit of avoiding other common allergens that provoke symptoms such as cat or dog hair, with no clear benefit to asthma symptom detected (Wood, Chapman et al. 1989, Wood, Johnson et al. 1998). The BTS guidelines do not recommend the routine use of allergen avoidance as secondary prevention in asthma particularly if focussed on a single agent. If a sensitized individual (or their family) are highly motivated to try an allergen avoidance strategy they recommend a multi-faceted approach which addresses HDM, moulds and all other indoor pollutants (Krieger, Takaro et al. 2005).

1.1.2 Immunotherapy

Subcutaneous allergen-specific immunotherapy

Subcutaneous allergen-specific immunotherapy (SCIT) involves injecting allergens of increasing amounts under the skin of a patient who is known to be sensitive to it in order to 'desensitise' them. It has been the subject of some controversy as positive results were unconvincing in early studies and there were concerns regarding the risk of fatal allergic reactions (Frew 1994, Creticos, Reed et al. 1996). A Cochrane review in 2010 considered 88 trials of injected immunotherapy for the treatment of chronic asthma (Abramson, Puy et al. 2010). The majority were for dust-mite allergy, other allergens considered were pollen, animal dander, Cladosporium and multiple allergies. Overall the studies showed significantly less use of asthma medications and improved asthma symptoms after immunotherapy. There was significantly reduced allergen-specific bronchial

hyperreactivity with immunotherapy and some reduction in general bronchial hyperreactivity. If 16 patients were treated with immunotherapy, 1 would be expected to develop a significant 'large local reaction' and if 16 were treated one would be expected to develop an adverse systemic reaction (of any severity). They recommended its use in those with severe difficult-to-control asthma who were sensitized to agents they were unable to avoid and recommended that the risks were carefully considered (Abramson, Puy et al. 2010).

Immunotherapy as primary prophylaxis is less well understood but several studies have demonstrated a decrease in new allergen sensitization in those undergoing immunotherapy compared to control (Pajno, Barberio et al. 2001, Purello-D'Ambrosio, Gangemi et al. 2001); however none of these had the scope to look at the development of clinical disease. One study by *Moller et al* looked at 205 children undergoing pollen immunotherapy for allergic rhinitis and demonstrated a lower rate of asthma development that persisted for a period of 3-4 years after treatment (Moller, Dreborg et al. 2002). More studies are required to clarify the position of SCIT as a primary prophylaxis for allergic disease.

Sublingual allergen specific immunotherapy

Due to the inconvenience and potentially serious risks associated with subcutaneous immunotherapy there has been considerable interest in developing an alternative delivery route that is equally efficacious with fewer potential risks. Sublingual immunotherapy (SLIT) is associated with a lower incidence of systemic reactions. There have been two systematic reviews of this therapy which demonstrated, at beast, a modest benefit to asthma control with SLIT but have not shown any significant improvement in asthma and allergy symptoms or medication use (2014)

A meta-analyses of SLIT against HDM showed some benefit in symptoms and medication use in children but not in adults. Again, due to variation in asthma scoring systems and symptom reporting, the magnitude of this benefit was difficult to quantify (2014)

1.1.3 Asthma Initiation

A history of asthma in the immediate family is known to be one of the biggest risks for a child developing asthma. The status of the mother has been regarded as most important either due to a genuine parent-of-origin effect or via influences of maternal exposure in pregnancy and immune interaction *in utero* (Moffatt and Cookson 1998, Matson, Zhu et al. 2007). Several studies have assessed maternal or paternal influence on childhood allergic disease with contradictory results. Though maternal asthma is thought to confer a high risk of asthma to a child (Litonjua, Carey et al. 1998, Withers, Low et al. 1998, Burke, Fesinmeyer et al. 2003, Lim, Kobzik et al. 2010) some studies show a greater paternal effect (Dold, Wjst et al. 1992) and others no difference (Bjerg, Hedman et al. 2007). An article by *Arshad et al* looking at the effect of parental influences in the Isle of Wight cohort demonstrated that both maternal and paternal asthma were consistently associated with increased risk of childhood asthma at all ages (Arshad, Karmaus et al. 2012). Paternal asthma significantly increased asthma risk in males but not in females and maternal asthma tended to increase asthma risk in females. A biparental asthma history had a greater effect on asthma risk and was equally important in males and females.

Longitudinal studies have shown that most asthma develops in childhood (Sly, Boner et al. 2008); but not all wheezing in infancy goes on to become asthma. As has been mentioned previously the diagnosis of asthma is not straightforward and often requires assessment over a period of time. Therefore asthma in young children can be extremely difficult to diagnose with confidence.

Suboptimal foetal growth, maternal malnutrition, allergy and smoking are all predictors of BHR and later asthma (Lucas, Inskip et al. 2004, Shaheen, Newson et al. 2004, Litonjua, Rifas-Shiman et al. 2006, Turner and Devereux 2007). Repeated rhinovirus (RV) infection during early childhood has been shown to increase the risk of developing asthma by the age of 6 by 26-fold (Jackson, Gangnon et al. 2008). The Isle of Wight Cohort Study demonstrated an adjusted risk of asthma age 10 was 4-fold in those who had recurrent chest infections before the age of 2 years (Arshad, Kurukulaaratchy et al. 2005, Raza, Kurukulaaratchy et al. 2008). A USA cohort study demonstrated that being born during the winter virus season conferred a 30% increased risk of developing asthma by the age of 6

years (Wu, Dupont et al. 2008) and infection with RV or respiratory syncytial virus (RSV) has been shown to positively interact with atopy to increase the likelihood of asthma by age 5 years (Turato, Barbato et al. 2008).

An interesting article arising from the Scandinavian birth cohort by *Sigurs et al* suggested that the severity of RSV infection is the important factor and found that severe RSV bronchiolitis (defined as the need to be hospitalised with the disease within the first year of life) was associated with increased risk of persistent wheeze and allergic sensitization at age 3 years (Sigurs, Bjarnason et al. 1995). At 7.5 years 23% of the severe RSV group had asthma compared with only 2% of the control group. Similar differences were seen when they were assessed at 13 years. The control group had antibodies to RSV measured at 1 year of life and 42% had demonstrable levels, suggesting that infection alone is not sufficient to cause asthma.

The Tuscon Children's Respiratory Study demonstrated that RSV infection was associated with wheeze at 11 years of age but not at 13 years (Stein, Sherrill et al. 1999) (not differentiating between severe and mild infection). Other studies have not demonstrated an association between RSV infection and asthma or allergic sensitisation in adolescence or adulthood. Again these studies have not differentiated between severe and mild infection (Pullan and Hey 1982, Korppi, Piippo-Savolainen et al. 2004). The answer is only likely to be conclusively answered with a controlled trial where preventing RSV infection results in a decrease (or not) of asthma incidence.

1.1.4 Asthma Transition During Adolescence

Asthma is a chronic relapsing-remitting disease which can follow a variable course from childhood into young adult life with some adolescents outgrowing their disease while others maintain disease into adulthood (Vonmutius 1996, Nicolai, Illi et al. 2001, Nicolai, Pereszlenyiova-Bliznakova et al. 2003, Vonk, Postma et al. 2004). A switch from male predominance before adolescence to female predominance afterwards has been described in several populations (Nicolai, Pereszlenyiova-Bliznakova et al. 2003, Mandhane, Greene et al. 2005, Tollefsen, Langhammer et al. 2007, Almqvist, Worm et al. 2008). Most studies

have also reported a net decrease in asthma during adolescence i.e. the rate of remission is greater than that of new asthma development. This was seen in the Tuscon Children's Respiratory Study (Guerra, Wright et al. 2004), the UK National Birth Cohort (Anderson, Pottier et al. 1992) and others (Nicolai, Pereszlenyiova-Bliznakova et al. 2003, Bronnimann and Burrows 2009). Although there may be a slight increase in male asthma remission during adolescence according to some studies (Sekerel, Civelek et al. 2006) and not in others (Nicolai, Illi et al. 2001, de Marco, Locatelli et al. 2002, Burgess, Matheson et al. 2011); it is widely accepted that this 'sex reversal 'is predominantly due to an increase in the prevalence of mainly non-atopic female airways disease during this period (Becklake and Kauffmann 1999, Nicolai, Pereszlenyiova-Bliznakova et al. 2003, Mandhane, Greene et al. 2005, Tollefsen, Langhammer et al. 2007, Burgess, Matheson et al. 2011). The age at which this switch occurs is not known but the TRAILS study demonstrated that by the age of 11.1-years the incidence was equal in males and females and by the age of 16.3 years significantly higher in females than in males (Vink, Postma et al. 2010). A Canadian study looked at 20 277 subjects with first diagnosis of asthma who were re-admitted to hospital for asthma and discovered that there was little sex difference between age 1 and 9-years but markedly higher asthma in female than males between the ages of 10 and 19-years (Chen, Dales et al. 2003).

During adolescence many young people will also grow out of asthma. Rates of between 50 to 65% have been reported for asthma remission (Burgess, Matheson et al. 2011); and although this 'remission' can be complete, asthma may return in a significant proportion, and relapse later in life is common (Sears, Greene et al. 2003). A community-based cohort study in Melbourne reported a remission rate of 55% between the ages of 7 and 21 years but a relapse rate of 25% by the age of 42 years (Martin, Mclennan et al. 1980, Phelan, Robertson et al. 2002). A large population-based Swedish study reported a 10 year remission rate of 14.6% (Ekerljung, Ronmark et al. 2008). The male-female reversal of asthma incidence during adolescence has been studied to see if it is due to an increased rate of remission among boys, an increased incidence among girls or a combination of both. Remission is more likely in those whose asthma began before the age of 10 years and less likely in those exposed to passive smoking and of lower socioeconomic status (de Marco, Locatelli et al. 2000, Burgess, Matheson et al. 2011). The Tuscon study demonstrated that

remission was 3.7 as likely in childhood-onset asthma and 1.3 times as likely in adolescent onset asthma as adult-onset asthma. Allergic rhinitis, childhood chronic bronchitis and (to a lesser extent) eczema have also been shown to have a negative effect on remission (Ekerljung, Ronmark et al. 2008, Burgess, Matheson et al. 2011) and remission appears to be less likely in those who have decreased lung function at age 7 years (de Marco, Locatelli et al. 2002, Burgess, Matheson et al. 2011). However, in many asymptomatic teenagers who have 'grown out' of asthma there still remains BHR, airway inflammation and thickening of the lamina reticularis (Van den Toorn, Overbeek et al. 2001, Obase, Shimoda et al. 2003, Hara, Fujimura et al. 2008). It is possible that residual bronchial hyper-reactivity can predict relapse of clinical asthma later in life.

The relationship between asthma and smoking in adolescence is complicated. While in later life several years of smoking seems to confer a negative effect on remission, in adolescence there appears to be a positive relationship between smoking and remission (Vonk, Postma et al. 2004, Burgess, Matheson et al. 2011). This has been suggested to be due to the 'healthy smoker' effect where those with healthy lungs are more likely to take up smoking (Becklake and Lalloo 1990). Presumably there would also be more parental pressure not to smoke in those with asthma which is likely to be more relevant in young adults who live at home than older adults.

Several factors have been linked to asthma persistence; including early-onset persistent wheezing, disease severity, reduced lung function and sensitization to multiple allergens and allergic co-morbidity (Phelan, Robertson et al. 2002). Other factors linked to developing, exacerbating and prolonging asthma are biologically active agents such as air pollutants, tobacco smoke and respiratory viruses (Sears, Greene et al. 2003, Sly, Boner et al. 2008). Analysis of persistent wheezers in the Isle of Wight cohort identified similar factors, finding that asthmatics family history, atopic skin prick testing age 4 years and recurrent chest infections at 2 years showed independent significance for persistence (Kurukulaaratchy, Matthews et al. 2003).

Given the positive effect of atopy on asthma persistence it would seem reasonable to suggest that if atopy remits, then asthma would be more likely to also. Remission of atopy has been linked to an 'immune tolerance' effect which has been targeted via the

administration of SLIT or SCIT. Immune tolerance to allergens is a shift in the balance of cytokine secretions away from the allergic T_H2 pattern towards the T_H1 atopy-inhibitory profile (Durham, Ying et al. 1996, Hamid, Schotman et al. 1997). This can be induced by repeated low-dose exposure to allergens and is thought to be associated with T-cell anergy whereby the T-cells responding to the allergen are actively supressed. A meta-analysis of SLIT treatment in an allergic paediatric population demonstrated reductions in asthma symptoms and medication use (Penagos, Passalacqua et al. 2008).

Our current increasingly-urbanised society, combined with attempts during early life to 'protect' children from allergen exposure may have denied them the opportunity to develop tolerance via regular exposure to low doses of everyday allergens. This lack of tolerance could be a factor that contributes negatively to the remission of asthma.

1.1.5 Gender Differences in Asthma

Adolescence is a time of important physiological change that may contribute to sex-specific differences in allergic disease prevalence during that period. It has been postulated that the sex differences in allergic disease during adolescence may be influenced by a combination of biological sex differences, socioeconomic and environmental differences or epigenetic factors from the prenatal period which are influenced by this period of intense physiological development.

Sex hormones

Female sex hormones have been linked to the development of asthma during adolescence and the risk of asthma has been shown to be increased two-fold in those with early onset menarche (Al-Sahab, Hamadeh et al. 2011). This makes the assumption that an earlier menarche means longer exposure to female sex hormones. However a study by *Vink et al* (Vink, Postma et al. 2010) failed to show an association between asthma and pubertal stages, although in this study the period of follow-up was only to 16 years and it could be argued that not all adolescents will have completed puberty at this age.

Hormonal changes during puberty have also been implicated in eczema with some evidence to suggest that oestrogens may be pro-inflammatory and androgens protective against allergic inflammation (Chen, Mempel et al. 2008, Torres-Borrego, Molina-Teran et al. 2008).

Obesity

Obesity rates in the Western World are a considerable health concern particularly in the younger population (Eder, Ege et al. 2006, Moraes, Beltran Rosas et al. 2006). Obesity has been linked to asthma and its development, particularly in childhood (Suglia, Chambers et al. 2011) and in adults (Haldar, Pavord et al. 2008, Moore, Meyers et al. 2010) but information about the adolescent period has been conflicting. The Tuscon birth cohort demonstrated that becoming overweight between the age of 6 and 11 years increased the risk of developing asthma symptoms and increased bronchial hyper-reactivity in adolescence (Castro-Rodriguez, Holberg et al. 2001). In the Canadian National Population Health Survey a raised body mass index (BMI) was positively associated with adolescent asthma development in females but not in males (Chen, Mempel et al. 2008). The Dunedin cohort estimated that 28% of asthma developing in females after the age of 9 years can be attributed to being overweight (Hancox, Milne et al. 2005). A more recent study from *Yoo et al* had a somewhat contradictory finding that that overweight adolescent boys (not girls) had increased risk of wheeze and atopy (Yoo, Kim et al. 2011).

Fat related hormones e.g. adipokines and leptin are currently under scrutiny for possible relationship with asthma although conclusive results are not yet apparent. High leptin levels have been linked with bronchospasm (Baek, Kim et al. 2011) while high adiponectin levels seem to have some protective effect in males (Kattan, Kumar et al. 2010). Other studies have failed to show any association between these 'obesity hormones' and asthma (Sutherland, Sears et al. 2009).

Environmental factors

Some studies have reported a difference in susceptibility to environmental factors between males and females. This has been suggested to explain the stronger association between exposure to gas cooking and respiratory symptoms in girls than boys, the increased

vulnerability to the effects of ozone and the more marked effect of inhaled tobacco smoke on lung function (Martinez, Antognoni et al. 1988, Messineo and Adams 1990, Jarvis, Chinn et al. 1998).

1.2 INFLAMMATION

1.2.1 The Inflammatory Cascade

Asthma has traditionally been described as a disease of acute on chronic inflammation characterised by bronchial hyper-reactivity. Immunohistochemical analysis of bronchial biopsies has shown activation of inflammatory pathways with release of multiple cytokines, chemokines and growth factors.

1.2.1.1. Acute and late-phase response

After the initial sensitization to an allergen (covered in previous chapter) re-exposure to the allergen results in an inflammatory reaction in the asthmatic airways. The early response commences when the inhaled allergen binds to preformed IgE which leads to activation of mast cells and macrophages (Tonnel, Gosset et al. 1983, Calhoun, Reed et al. 1992). These cells release proinflammatory mediators such as histamine which lead to mucous secretion, smooth muscle contraction and vasodilation (Jarjour, Calhoun et al. 1997). An additional effect on the bronchial microvasculature is the stimulation of plasma protein leakage which results in a thickened oedematous airway lumen, exacerbating the airway narrowing caused by smooth muscle contraction and thus potentiating asthma symptoms (Greiff, Erjefalt et al. 1993, Vanvyve, Chanez et al. 1995). The combination of plasma protein, mucus, epithelial cells and inflammatory cells promote the formation of the viscid mucous plugs often recognised in cases of fatal asthma as far back as the 1920s (HL 1922, Dunnill 1960).

The late-phase inflammatory reaction involves recruitment and activation of eosinophils, CD4+ T cells, basophils, neutrophils and macrophages and the polarisation of T lymphocytes towards a T_H2 profile (type 2 CD4+ T cells) (Demonchy, Kauffman et al. 1985, Calhoun, Jarjour et al. 1993, Koh, Dupuis et al. 1993, Robinson, Hamid et al. 1993, Guo, Liu et al. 1994, Montefort, Gratziou et al. 1994). There is also release of cytokines IL-3, IL-4, IL-5, IL-9, IL-13, IL-17 and GM-CSF (granulocyte macrophage colony stimulating factor) (Howarth, Wilson et al. 1991). Not all asthma, however, involves allergic sensitisation; those with non-allergic asthma (not driven by IgE antibodies) share many features with allergic asthma via the T_H2 pathway (Wills-Karp 1999). Although T_H2 cells are the important mediator pathway in mild to moderate asthma, in recent years it has been demonstrated

that as asthma becomes more severe and chronic T_H1 cells begin to have a more predominant role, possibly via the secretion of interferon gamma (IFN-γ) which inhibits T_H2 cell proliferation (Afshar, Medoff et al. 2008).

1.2.1.2. Chronic inflammation

The persistence of any inflammatory response is regulated by apoptosis, a dynamic process which controls the 'tissue load' of cells at any site of inflammation. In healthy tissue this process tends to promote resolution rather than persistence of inflammation (Haslett, Savill et al. 1994, White 1996). There is some evidence to demonstrate reduced apoptosis and increased expression of adhesion molecules on epithelial cells in the airways of asthmatic subjects (Vignola, Campbell et al. 1993, Canonica, Ciprandi et al. 1994, Simon and Blaser 1995, Woolley, Gibson et al. 1996, Walsh 1997). There is also over-expression of cytokines that promote cells survival such as GM-CSF, IL-3, IL-5 and RANTES (regulated on activation, normal T cell expressed and secreted) (Robinson, Ying et al. 1993, Sousa, Poston et al. 1993, Woolley, Adelroth et al. 1994, Humbert, Ying et al. 1997). Glucocorticoids reduce the survival of inflammatory cells such as eosinophils (Wallen, Kita et al. 1991, Meagher, Cousin et al. 1996).

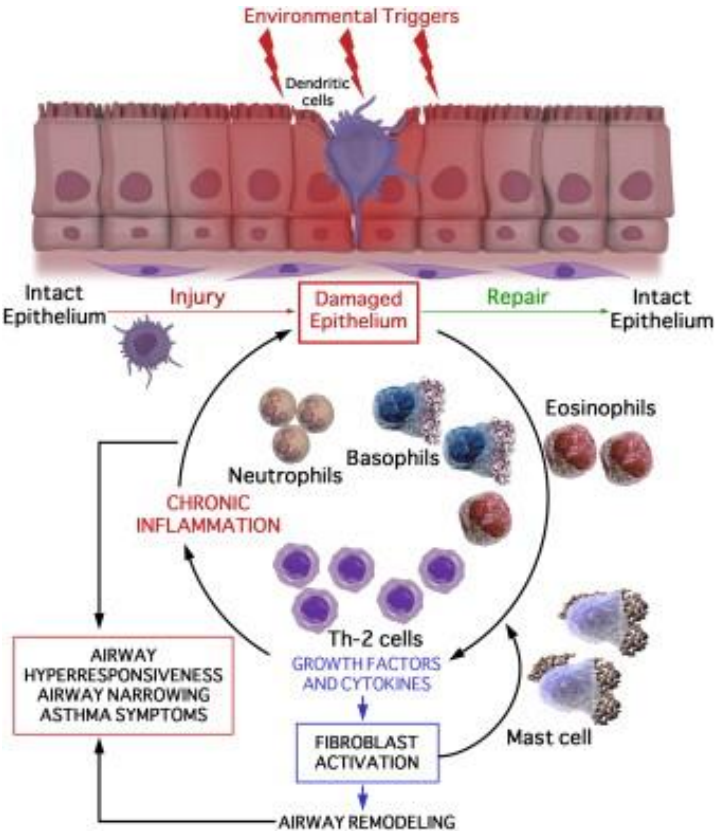


Figure 2a: Schematic representation of how epithelial injury and aberrant repair interact with inflammatory components in chronic asthma.
(Permission to reprint requested and granted by Journal of Allergy and Clinical Immunology, Vol 120, (6) 2007, p1233 – 1244)

1.2.2 Inflammatory Cells in Asthma

1.2.2.1 Eosinophils

Tissue eosinophilia is seen in both allergic and non-allergic asthma and is present even in mild disease. While it is characteristic of asthma it can also be seen in other diseases (Lacoste, Bousquet et al. 1993). In symptomatic asthma these eosinophils are activated (Broide, Gleich et al. 1991, Laitinen, Laitinen et al. 1991) and a correlation between eosinophil activation and severity of disease (Bousquet, Chanez et al. 1990), as well as BHR (Bradley, Azzawi et al. 1991), has been demonstrated. Allergen challenge leads to

recruitment and activation of eosinophils as part of the late phase response (Kroegel, Liu et al. 1994, Shaver, Zangrilli et al. 1997). This correlates with soluble VCAM-1 (vascular adhesion molecule) levels and the production of IL-4 and IL-5 (Zangrilli, Shaver et al. 1995).

Within airway tissue eosinophils produce toxic granule proteins, oxygen free radicals, eicosanoids (Busse and Sedgwick 1994), T_H2-like cytokines (Broide, Paine et al. 1992, Ying, Durham et al. 1995) and growth factors (Weller, Moses et al. 1991). This leads to a number of effects on the airways including smooth muscle contraction (Rabe, Munoz et al. 1994), increased vascular permeability (Collins, Dupuis et al. 1993) and increased BHR (Leff 1994). They also have a harmful effect on the airway epithelium via the release of major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and oxygen free radicals which cause the epithelial shedding that is characteristic of asthma (Gleich, Adolphson et al. 1993). The role of eosinophils in remodelling is not fully understood but they appear to be involved in the fibrotic process seen in pulmonary fibrosis (Schlick 1993). Their ability to release growth factors (Ohno, Lea et al. 1992, Walz, Nishikawa et al. 1993), elastase (Lungarella, Menegazzi et al. 1992) and metalloproteinases (Ohno, Ohtani et al. 1997), all of which are part of the remodelling process, suggests that they are likely to be involved in the process of remodelling and fibrosis in asthma.

1.2.2.2 Mast cells

Mast cells are found in the airways of both normal subjects and asthmatics (Djukanovic, Wilson et al. 1990, Koshino, Arai et al. 1995). They are degranulated in asthmatic airways (Roche, Beasley et al. 1989) and produce mediators such as histamine, platelet derived growth factor 2 (PDG2) and cysteinyl leukotrienes which appear to act as a trigger for acute bronchoconstriction, airway wall oedema and mucous secretion. However a consistent link between mast cell degranulation and severity of asthma has not been established (Bousquet, Chanez et al. 1991, Jarjour, Calhoun et al. 1991) and only one study has shown a correlation between mast cell density in bronchial tissue and BHR (Koshino, Arai et al. 1995). Mast cells also release neutral proteases such as tryptase (Schwartz 1992), which potentiates histamine-induced contraction of lung tissue, and chymase, which has a procollagen proteinase effect (Kofford, Schwartz et al. 1997, Welle 1997).

Mast cells also play an important role in pulmonary fibrosis which has led to the suggestion that they may play a part in the fibrosis element of remodelling seen in asthma (Kawanami, Ferrans et al. 1979, Chanez, Lacoste et al. 1993, Jordana 1993). Human mast cells can release components of the basement membrane such as laminin and collagen IV (Thompson, Burbelo et al. 1991) as well as angiogenic growth factors (Meininger and Zetter 1992). They are also able to influence fibroblasts either by direct stimulation or via the action of tryptase which can stimulate collagen messenger ribonucleic acid (RNA) synthesis and fibroblast chemotaxis (Gruber, Kew et al. 1997).

1.2.2.3 Neutrophils

While eosinophils have long been identified as the characteristic cell in most types of asthma, more recently the role of neutrophils in asthma has been discussed. Neutrophils have been found to be present in very high numbers in pathology samples from the airways of those who have died from fatal asthma (Sur, Crotty et al. 1993), in particular those who died within a short time from commencement of attack. This is thought to be due to the fact that he longer the duration of symptoms the more likely that eosinophilic inflammation would predominate. Neutrophils are increased in biopsies from those with severe and corticosteroid-dependent asthma compared to those with mild asthma (Wenzel, Szefler et al. 1997, Jatakanon, Uasuf et al. 1999, Wenzel, Schwartz et al. 1999). A study by *Choi et al* demonstrated that neutrophilic inflammation in asthma was associated with persistent airways obstruction whereas predominantly eosinophilic inflammation was not (Choi, Jang et al. 2012). Whether they have a role in mild to moderate stable asthma is unclear.

1.2.3 Therapeutics and the Inflammatory Cascade

1.2.3.1 Corticosteroids and long-acting β_2-adrenoceptor agonists

Traditional asthma treatment has been with corticosteroids which are thought to suppress the T_H2 cytokine response and inhaled corticosteroids have been shown to have a beneficial effect on BHR in mild to moderate asthma (Olivieri, Chetta et al. 1997). There is, however, a significant proportion of patients with chronic asthma who have persistent

symptoms despite high-dose inhaled corticosteroids (Shrewsbury, Pyke et al. 2000). This would suggest that inflammation is not the only factor at play in chronic asthma.

In the clinical setting long-acting β_2-adrenoceptor agonists have been used as a conjunctive therapy to corticosteroids; these work to relax underlying smooth muscle and act synergistically with the inhaled corticosteroid. In 1994 *Greening et al* demonstrated an increase in peak expiratory flow rate (PEFR) in those who were prescribed a long-acting β_2 agonist in addition to their inhaled steroid compared with those who were prescribed an increase in the steroid dose.(Greening, Ind et al. 1994) There was no difference in adverse outcomes at 6 months, suggesting that the lower corticosteroid dose was not associated with longer-term loss of asthma control.

1.2.3.2 Anti-IgE

IgE is known to be a key mediator of allergic diseases such as asthma and rhinitis (Sutton and Gould 1993, Platts-Mills 2001). It is produced by B cells after sensitization (Bousquet, Vignola et al. 2003) and has a short half-life (MacGlashan, Lichtenstein et al. 1999). It is highly immunologically active due to the presence of high-affinity receptors on mast cells and basophils (Bousquet, Vignola et al. 2003). IgE binds to these receptors and cross-links are formed between the allergen and IgE molecule and the inflammatory cascade is activated via release of mediators such as leukotrienes, histamine and platelet activating factor (Arshad and Holgate 2001). IgE is thought to be involved in the initiation and perpetuation of both allergic and non-allergic asthma (Beeh, Ksoll et al. 2000, Ying, Humbert et al. 2001, Powe, Jagger et al. 2003). Almost all forms of asthma, including occupational asthma (Lombardo and Balmes 2000), are associated with elevated IgE levels (Burrows, Martinez et al. 1989) as are other conditions such as allergic rhinitis (Bush 2004).

Omalizumab is a recombinant humanized monoclonal anti-IgE antibody (mAb). It forms complexes with IgE by binding to the Fc part of the IgE antibody forming an IgE-Omalizumab complex and blocks the interaction between IgE and mast cells and basophils that drives the IgE-mediated component of allergic disorders such as asthma (Schulman 2001). It inhibits markers of inflammation such as mast cell degranulation, histamine release, pulmonary eosinophilic infiltration (Shields, Whether et al. 1995, Coyle, Wagner et al. 1996, Boulet, Chapman et al. 1997, Fahy, Fleming et al. 1997, Milgrom, Fick et al. 1999) and

exhaled nitric oxide (Silkoff, Milgrom et al. 2000). Bronchial biopsies before and 12 weeks after Omalizumab treatment showed an 80% reduction in airway mucosal eosinophils and almost complete loss of many components of the inflammatory cascade, including mast cells, basophils, dendritic cells, CD 4, CD8 and CD20 B cells (Djukanovic, Wilson et al. 2004). Although one single dose of Omalizumab has been shown to rapidly reduce free IgE levels in patients with allergic asthma by over 95% (Boulet, Chapman et al. 1997, Corne, Djukanovic et al. 1997), the very high density of receptors present on effector cells would suggest that a 99% reduction in circulating IgE would be necessary to block IgE mediated inflammatory reactions (MacGlashan, Bochner et al. 1997). Omalizumab has been shown to have a second mode of action which is to down-regulate receptor expression on basophils (Saini, MacGlashan et al. 1999, Beck, Marcotte et al. 2004); it is thought that this is related to reduced circulating levels of free IgE (Saini, MacGlashan et al. 1999). Omalizumab has also been shown to down-regulate high-affinity IgE receptors on dendritic cells suggesting that it also has a role in altering allergen presentation which is a critical step in the allergic response (Feuchtinger, Bartz et al. 2003, Prussin, Griffith et al. 2003, Hayek, Laimer et al. 2004).

Omalizumab has been demonstrated to reduce both the early and late asthmatic response (Boulet, Chapman et al. 1997, Fahy, Fleming et al. 1997) and show a significant increase in FEV1 after allergen challenge during both phases (Fahy, Fleming et al. 1997). It also reduces the number of IgE+ and high affinity IgE receptor cells in the bronchial submucosa and epithelium of patients with asthma (Djukanovic, Wilson et al. 2004). This is thought to be of particular importance given that the presence of high–affinity IgE receptors in patients with asthma is associated with a three-fold increase in mortality (Fregonese, Swan et al. 2005).

Several studies have now demonstrated the efficacy of Omalizumab in patients with severe difficult to control allergic asthma (Busse, Corren et al. 2001, Soler, Matz et al. 2001, Buhl, Soler et al. 2002, Ayres, Higgins et al. 2004, Holgate, Chuchalin et al. 2004, Vignola, Humbert et al. 2004) including the INNOVATE (The Investigation of Omalizumab in seVere Asthma TrEatment) (Humbert, Beasley et al. 2005). A pooled analysis of Omalizumab studies by *Bousquet et al* found that Omalizumab significantly reduced exacerbation rates and emergency visit rates compared to control (Bousquet, Cabrera et al. 2005). There were

also a significantly increased quality of life scores in those using Omalizumab (Buhl, Hanf et al. 2002, Finn, Gross et al. 2003, Humbert, Beasley et al. 2005).

1.2.4 New targets for Therapeutics

1.2.4.1 IL-5

Another cytokine, IL-5, has been identified in animal studies as particularly relevant to asthma due to its effect on eosinophil development and priming. IL-5 recruits eosinophils from bone marrow and promotes their activation and persistence (Samitas, Radinger et al. 2011, Walsh 2012).

Mepolizumab is a humanized monoclonal antibody that acts on IL_5 and has been shown to reduce circulating (90%), sputum (60-80%) and tissue (55%) eosinophils. It has been used as a glucocorticoid sparing agent in severe eosinophilic asthma and has been shown to reduce asthma exacerbations in this group of patients (Haldar, Brightling et al. 2009, Pavord, Korn et al. 2012).

In the New England Journal of Medicine published trial SIRIUS (Steroid Reduction with Mepolizumab Study) *Pavord et al* demonstrated that a lower dose of Mepolizumab given subcutaneously to patients with elevated blood eosinophils. They demonstrated a reduction in glucocorticoid dose and also an improvement in asthma symptoms, exacerbations and quality of life despite this reduction in medication (Bel, Wenzel et al. 2014). In light of this Mepolizumab may represent a treatment option for those with glucocorticoid-dependent severe eosinophilic asthma in the future.

1.2.4.2 IL-4 AND IL-13

IL-4 and IL-13 are structurally homologous cytokines which due to their effects on epithelial cells and fibroblasts have been identified as potentially important with regards to airway wall remodelling in transgenic animal models (Shirakawa, Deichmann et al. 2000). They have overlapping but not identical effector properties as they both use the same receptor α chain (IL-4Rα). Both of these interleukins signal via the transcription factor STAT-6 (signal

transducer and activator of transcription 6) which has been shown to be prominent on the bronchial epithelium particularly in severe asthmatics (Mullings, Wilson et al. 2001).

Overexpression of IL-13 in bronchial epithelium of mice causes lymphocytic and eosinophilic infiltration, goblet cell metaplasia, subepithelial fibrosis and increased smooth muscle; this is associated clinically with increased BHR (Laporte, Moore et al. 2001, Grunstein, Hakonarson et al. 2002). Production of IL-13 is inhibited by inhaled corticosteroids (ICS) but severe asthmatics continue to have high levels of IL-13b despite high-dose of oral corticosteroids and ICS (Saha, Berry et al. 2008); this led to the hypothesis that IL-13 could contribute to corticosteroid resistance (Spahn, Szefler et al. 1996, Kraft, Hamid et al. 2001, Hakonarson, Bjornsdottir et al. 2005, Barnes and Adcock 2009).

Corren et al conducted a randomised, double-blind, placebo-controlled study of lebrikizumab, an IgG4 humanized monoclonal antibody that specifically binds to IL-13 and inhibits its function (Corren, Lemanske et al. 2011). They looked at patients with severe, difficult-to-control asthma and evidence of high IL-13 activity despite being on high doses of both inhaled and oral corticosteroids. Periostin was used as a surrogate marker for IL-13 as its production from epithelial cells has been demonstrated to be induced by IL-13 (Sidhu, Yuan et al. 2010) and IL-13 activity requires highly sensitive assays (St Ledger, Agee et al. 2009). The primary outcome was change in FEV_1 (forced expiratory volume in 1 second) and secondary outcome rate of asthma exacerbations over the 24 week period. They demonstrated a significant increase in pre-bronchodilator FEV_1 in those with high periostin levels taking the lebrikizumab versus placebo. There was a trend towards lower exacerbation rate in the treatment group which did not reach statistical significance and no difference in asthma symptom scores or use of reliever medication. Benefit seemed to be greater in those with high Periostin levels (Corren, Lemanske et al. 2011).

1.2.4.3 IL-17

IL-17 is a relatively newly identified cytokine which seems to be of significance in asthma at the severe end of the spectrum; the severity of BHR correlates with L-17 levels (Chakir, Shannon et al. 2003). There are three subtypes: IL-17A, IL-17E and IL-17F. It has been demonstrated that the presence of TGF-β and IL-6 combined causes T-helper cells to differentiate into IL-17 producing T_H17 cells (Bettelli, Carrier et al. 2006, Mangan,

Harrington et al. 2006). IL-17 is also produced by natural killer T cells (Michel, Keller et al. 2007), neutrophils (Li, Huang et al. 2010) and macrophages (Song, Luo et al. 2008). It is expressed on lung epithelial cells after exposure to allergens (Hurst, Muchamuel et al. 2002, Angkasekwinai, Park et al. 2007, Hammad, Chieppa et al. 2009) and has been detected in bronchial submucosa of asthmatic subjects (Letuve, Lajoie-Kadoch et al. 2006). IL-17 acts as a neutrophil chemoattractant (Roussel, Houle et al. 2010) and has been implicated in corticosteroid resistance in severe asthma. It may be a potential therapeutic target in a subgroup of severe neutrophilic asthmatics (Aujla and Alcorn 2011).

1.3 THE EPITHELIAL MESENCHYMAL TROPHIC UNIT

1.3.1 Epithelial Function

Every day the human lung surface, which has an estimated area of 100m^2, is exposed to approximately 10,000L of air. The epithelium covering this surface is the physical barrier between the lung tissue and harmful agents in the inhaled environment. The epithelium is covered with a thin layer of fluid, the mucociliary escalator, in which particles are trapped and transported out of the lungs with the aid of the peristaltic action of microvilli. Bronchial epithelium consists of ciliated columnar cells, mucous-secreting goblet cells and surfactant-secreting Clara cells; together they form an impermeable barrier through the formation of tight junctions (TJs). Tight junctions are made up of interacting proteins and receptors that allow communication between the cells and are able to regulate the transport of intercellular material (Roche, Montefort et al. 1993). The epithelium secretes a number of anti-microbial peptides such as defensins, cathelicidins and collectins which are toxic to pathogens (Evans, Xu et al. 2010). Epithelial-based receptors such as PARs and TLRs on the epithelium are primed to react quickly to a presenting microbial compound or environmental allergen. This leads to recruitment and activation of inflammatory cells via the release of cytokines therefore acting as an immunological barrier in addition to a physical one (Hammad and Lambrecht 2008, Parker and Prince 2011). There has been much interest in effect of these epithelial factors on the subepithelial layers both in non-disease and disease states.

1.3.2 The Asthmatic Epithelium

The asthmatic epithelium has been the focus of much interest and has been shown to be both structurally and functionally abnormal. Post-mortem samples of patients who had died due to their asthma showed sloughing of the columnar epithelium (epithelial shedding) and increased numbers of ciliated epithelial cell clumps (Creola bodies) (Hogg 1993). Similar evidence of epithelial shedding has been seen in bronchoalveolar lavage (BAL) fluid taken from severe asthmatics (Gleich 1986). Epithelial damage of this nature is not seen in most other chronic respiratory conditions such as cystic fibrosis and chronic obstructive pulmonary disease (COPD). Bronchial biopsies from asthmatic subject also

demonstrate possible mechanisms for this damage with increased expression of heat shock protein (a cell stressor) and activation of the caspase enzyme cascade involved in apoptosis (Bertorelli, Bocchino et al. 1998). *Bucchieri et al* demonstrated clumps of p85+ (a marker for early apoptosis) epithelial cells in lavage fluid and columnar cells on biopsy staining (Bucchieri, Puddicombe et al. 2002). Increased fas and fas-ligand expression in severe asthma and evidence of DNA fragmentation suggest that the asthmatic epithelium is more susceptible to injury than normal epithelium (Trautmann, Schmid-Grendelmeier et al. 2002).

The antioxidant pathways that help resist injury such as superoxide dismutase and glutathione peroxidase are also defective (Comhair, Bhathena et al. 2001, Bucchieri, Puddicombe et al. 2002). When cultured and exposed to an oxidant stimulus such as hydrogen peroxide (H_2O_2) asthmatic bronchial cells exhibit a three-fold increase in anexin 5 (a marker of apoptosis that identifies disruption of the plasma membrane) when compared with bronchial cells from normal subjects (Bucchieri, Puddicombe et al. 2002). These changes have been seen in childhood asthma suggesting that they are a key feature of the disease (Fedorov, Wilson et al. 2005).

Compared with cells from healthy controls, bronchial cells from asthmatic epithelium fail to form effective tight junctions when cultured in vitro; despite the fact that they have been separated from any airway inflammatory cells or mediators for at least 6 weeks (figure 2b). In addition transepithelial resistance (TER) is markedly reduced; indicating increased cell permeability. This becomes more marked if the cells are exposed to cigarette smoke, and the reduction is seen at a lower concentration than is necessary to effect the same change in non-asthmatic epithelium (Ilowite, Bennett et al. 1989). Asthmatic epithelium also shows increased permeability after exposure to ozone or nitrogen dioxide (Bayram, Rusznak et al. 2002) as has an increase in IL-8 and GM-CSF when exposed to diesel exhaust particles (Bayram, Devalia et al. 1998). Environmental allergens such as house dust mite allergen and respiratory viruses also have the capability to disrupt tight junctions and adversely affect epithelial permeability (Wan, Winton et al. 2000).

Figure 2b: Immunofluorescent confocal microscopy of whole mounts of bronchial biopsies from a normal *(left)* and asthmatic *(right)* airway. The section has been stained with an antibody that identifies the TJ protein ZO-1 *(green)*, with the epithelial cell nuclei stained *red*. Note the lack of TJ organization in the asthmatic epithelium when viewed in the horizontal and vertical planes.

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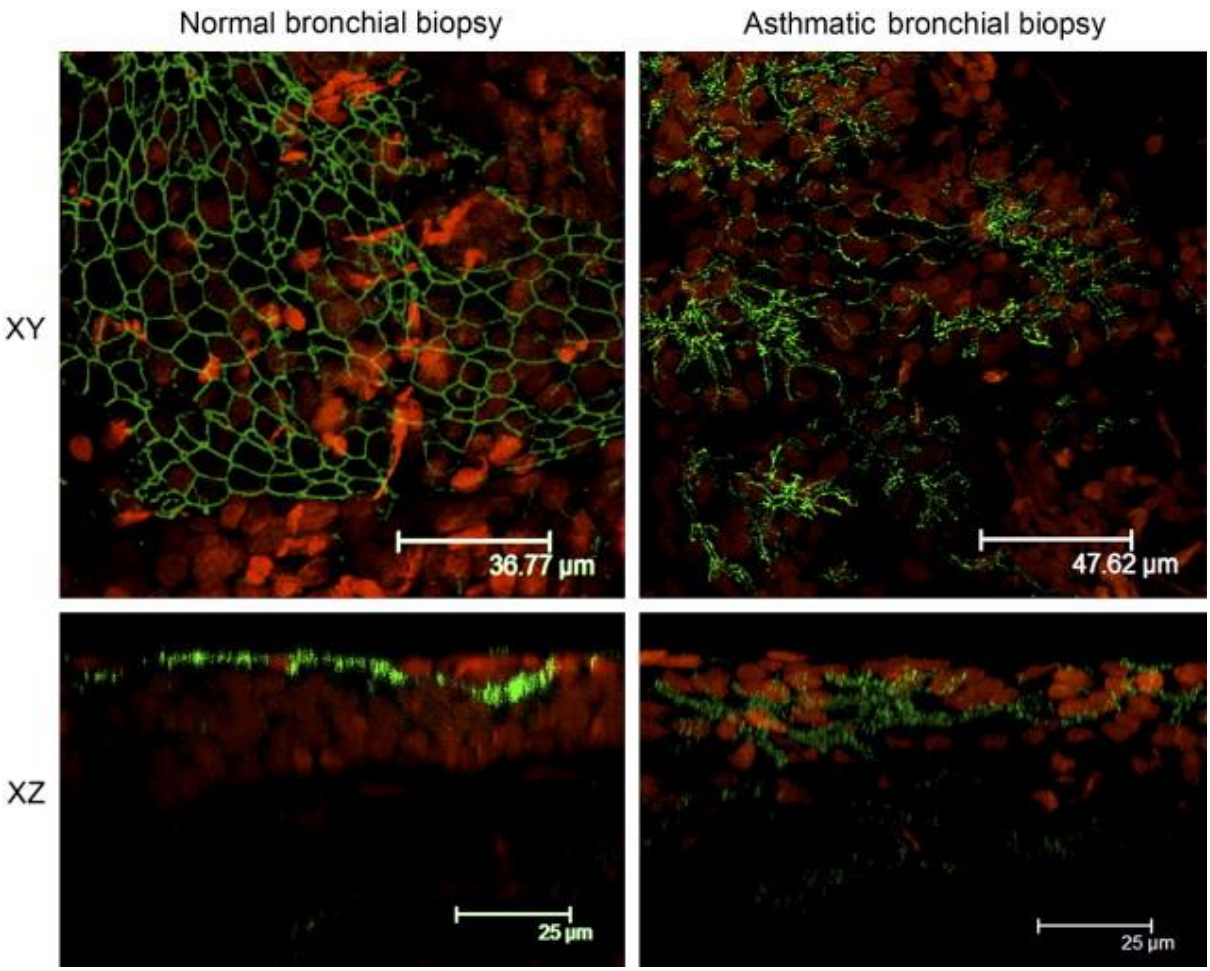
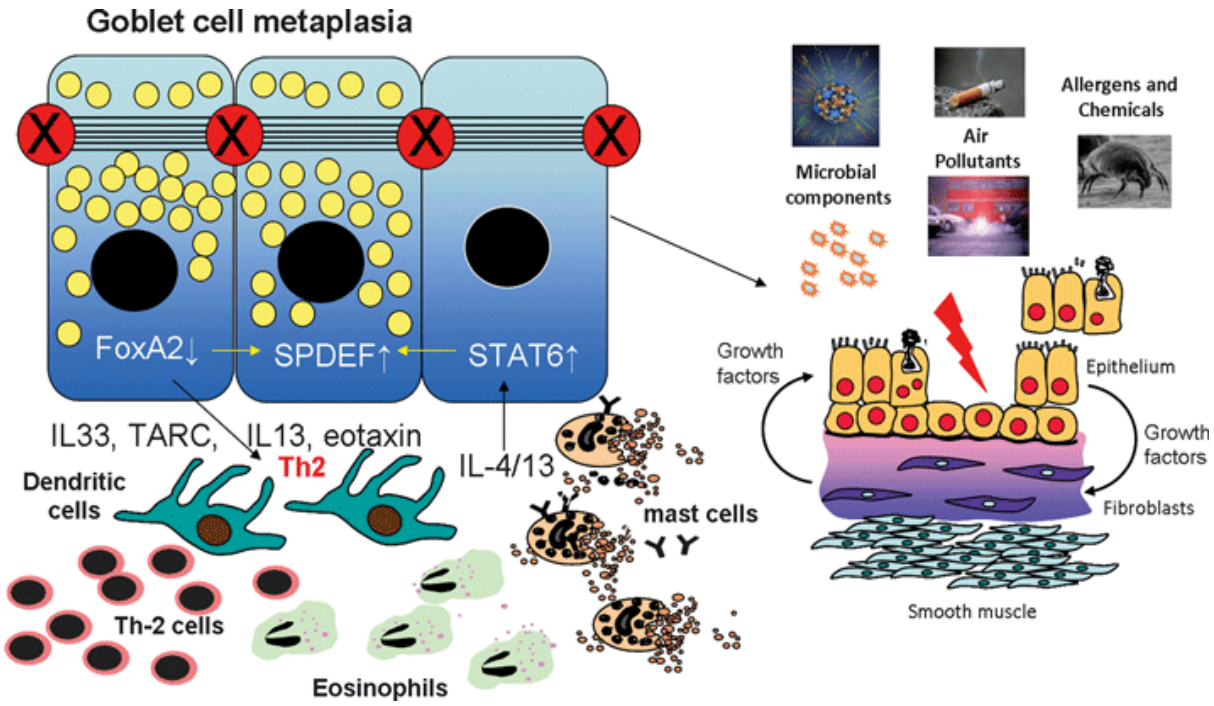


1.3.2.1 Abnormal Repair Mechanisms: Epidermal Growth Factor Receptor and p21^{waf}

The usual response of epithelium to injury is to upregulate epidermal growth factor receptors which drive proliferation and repair. Biopsies of asthmatic epithelium show increased numbers of these receptors proportional to disease severity, as one would expect as a response to epithelial injury (Amishima, Munakata et al. 1998, Puddicombe, Polosa et al. 2000). This increase is not, however, accompanied by an increase in markers of cell proliferation such as proliferating cell nuclear antigen (PCNA) even in severe asthmatics with marked evidence of epithelial shedding (Demoly, Simonylafontaine et al.

1994, Puddicombe, Torres-Lozano et al. 2003). This contrasts with findings observed in chronic bronchitis for example where epithelial damage leads to increased cell proliferation markers in an appropriate 'repair response'. This has been suggested to be related to the high expression of the cell cycle inhibitor $p21^{waf}$; which is counter-intuitively increased throughout the asthmatic epithelium especially in those with severe disease. This expression impairs proliferation by arresting cells at stage G1 of the cell cycle thus prolonging the epithelial repair stage (Puddicombe, Torres-Lozano et al. 2003). $p21^{waf}$ can be induced by TGF β_1 and β_2, which are known to be increased in asthmatic subjects. This seems to suggest that the asthmatic epithelium has a defective response to injury and is not able to effectively repair itself (Holgate, Davies et al. 2000) (figure 3).

Figure 3: Schematic representation of the sentinel role of the airway epithelium in coordinating mucous metaplasia and chronic airway inflammation.
(Permission to reprint requested and granted by Immunological Reviews, Vol 242, (1), p205 - 219, July 2011)



1.3.3 Epithelial-Mesenchymal Communication

Lung development in humans is controlled by transcription factors and signalling molecules. Branching morphogenesis of the airway is usually complete by week 25 of gestation, the alveoli start developing some time before the airway is complete (usually around 20 weeks gestation) and continue to do so until several years after birth. Communication between the epithelium and underlying mesenchyme has been well documented by *Alescio et al* (Alescio 1967). They demonstrated that when lung mesenchyme from the alveolar area was engrafted onto tracheal epithelium the tracheal epithelium began to show an alveolar cell phenotype and if lung epithelium was engrafted onto tracheal mesenchyme the epithelium would revert to a tracheal phenotype (Alescio 1967). This led to the suggestion of unidirectional signalling i.e. the mesenchyme produces signals that control the development of the epithelium by production of signalling factors such as transforming growth factor-β_3 (TGF-β_3) and fibroblast growth factor (FGF)-7 and -10. We now however know that the epithelium also produces signalling molecules (such as epidermal growth factor TGF-B_2) and that bidirectional signalling is likely to be a more representative model (Demayo, Minoo et al. 2002).

Injury to the asthmatic epithelium has been shown to cause release of fibroproliferative growth factors such as TGF-β_2 and other growth factors (such as fibroblast growth factor 2 and insulin-like growth factor-1) which promote fibroblast proliferation in the underlying mesenchymal layer (Zhang, Smartt et al. 1999). TGF-β_2 is of particular importance due to its ability to promote differentiation of fibroblasts into myofibroblasts that secrete interstitial collagen and growth factors (Richter, Puddicombe et al. 2001). At least two studies have described a layer of subepithelial cells with features of myofibroblasts correlating to the thickness of the reticular collagen layer in asthmatic subjects (Brewster, Howarth et al. 1990, Evans, Van Winkle et al. 1999). This layer would be ideally placed to communicate signals from the epithelium to the deeper submucosal layer.

Taking all of these findings into account a new model of asthma has been proposed whereby a genetically susceptible epithelium with aberrant repair mechanisms is exposed to damage by environmental agents and, unable to repair itself adequately, enters into a chronic inflammatory state through activation of the epithelial mesenchymal trophic unit

(EMTU). Signals are produced which act on the underlying mesenchymal cells thus propagating the inflammatory response and leading to smooth muscle proliferation and remodelling of the airway.

1.3.3 The Role of Epithelium-derived Cytokines in Asthma

1.3.3.1 IL-33

IL-33 was originally discovered as a nuclear factor of high endothelial venules (Baekkevold, Roussigne et al. 2003) and it is expressed by structural cells such as endothelial cells, bronchial smooth muscle, fibroblasts, keratinocytes and adipocytes (Schmitz, Owyang et al. 2005, Moussion, Ortega et al. 2008, Wood, Wang et al. 2009). It may also be produced by immune cells such as dendritic cells, macrophages and mast cells (Schmitz, Owyang et al. 2005). It is a member of the IL-1 cytokine family, all of which are involved in driving a different part of the inflammatory response (Guo, Wei et al. 2009). The IL-33 receptor is dimeric and consists of an IL-1R-like subunit (IL-1R1) which is associated with the IL-1R accessory protein. IL-1R1 is also known as ST2 which was identified as a marker of T_H2 cells before IL-33 was identified (Lohning, Stroehmann et al. 1998, Xu, Chan et al. 1998). Its receptor ST2 is expressed on vascular endothelial cells, epithelial cells, mast cells, eosinophils, basophils, natural killer T (NKT) cells and fully differentiated T_H2 cells (Aoki, Hayakawa et al. 2010). Unlike several other members of the IL-1 family which require cleaving to become activated it would appear that IL-33 is expressed and stored as a 30kDa full length precursor form (Funakoshi-Tago, Tago et al. 2011). It is thought to be released from the nuclei of cells in response to injury or necrosis and cleavage by caspase is thought to inactivate it (Cayrol and Girard 2009, Talabot-Ayer, Lamacchia et al. 2009, Hong, Bae et al. 2011).

Studies have found potent activities of IL-33 in mediating the T_H2 response. When IL-33 is administered systemically, mice produce increased T_H2 type cytokines and develop both airway eosinophilia and increased mucous production (Schmitz, Owyang et al. 2005). Airway administration of IL-33 markedly increases eosinophils in the airway and levels of IL-5 and IL-13 (Bartemes, Iijima et al. 2012) whereas administration of neutralizing

antibodies against either IL-33 or ST2 attenuates BHR and eosinophilia (Coyle, Lloyd et al. 1999, Liu, Li et al. 2009). Away from the field of asthma novel innate lymphoid cells that produce large amounts of T_H2 profile cytokines in response to IL-33 cells have been discovered in gastrointestinal organs such as mesenteric lymph nodes, spleen and mesentery (Moro, Yamada et al. 2010, Neill, Wong et al. 2010, Price, Liang et al. 2010, Saenz, Siracusa et al. 2010). Similar lymphoid cells have been seen in the lungs of mice infected with virus or exposed to allergens (Barlow, Bellosi et al. 2012, Bartemes, Iijima et al. 2012) thus further cementing a role for IL-33 in asthma.

Increased levels of IL-33 have been demonstrated in subjects with asthma (Prefontaine, Nadigel et al. 2010) and single-nucleotide polymorphisms in the IL-33 and ST2 genes have been associated with asthma (Gudbjartsson, Bjornsdottir et al. 2009). *Fujita et al* demonstrated that IL-33 induces IL-17F via ST2-ERK1/2-MSK1 signalling pathway in bronchial epithelial cells from human subjects (Fujita, Kawaguchi et al. 2012) giving rise to the possibility that the IL-33/IL-17F axis could be a possible therapeutic target.

1.3.3.2 IL-8

IL-8 is a chemokine belonging to the C-X-C subfamily which is involved in a wide variety of physiological and pathological processes, including host defence against bacterial infection, angiogenesis, arteriosclerosis, and autoimmune disorders of skin, bones, and joints (Harada, Mukaida et al. 1996). It has been found to have proangiogenic and tumorigenic properties. It is also a powerful chemoattractant for neutrophils (Kunkel, Standiford et al. 1991) and can also influence the migration of monocytes (Schroder, Mrowietz et al. 1987) and eosinophils (Shute 1994). It acts through G protein-coupled receptors CXCR1 (Holmes, Lee et al. 1991) and CXCR2 (Murphy and Tiffany 1991) on target cells. In neutrophils it mobilises intracellular Ca^{2+} and activates phospholipase C (Wu, Larosa et al. 1993) which induces shape change and exocytosis of stored proteins leading to the release of superoxide anion and hydrogen peroxide (Baggiolini, Dewald et al. 1994).

Elevated concentrations of IL-8 have been found in sputum, bronchoalveolar lavage fluid, and bronchial tissues of subjects with pulmonary diseases such as cystic fibrosis, COPD, bronchitis, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis (IPF) (Yamamoto, Yoneda et al. 1997, Gibson, Simpson et al. 2001). In more recent years a

subgroup of severe asthma has emerged as being corticosteroid-resistant and predominantly neutrophils driven and this has led to investigation of a possible link between IL-8 and severe asthma.

Govinderaju et al demonstrated that human airway smooth muscle cells (HASMC) would synthesise IL-8 in response to tumour necrosis factor (TNF)-α, IL-1-β and bradykinin, and also that HASMC express mRNA and protein for CXCR1 and CXCR2 (Govindaraju, Michoud et al. 2006). Stimulation of these receptors caused the anticipated changes in Ca^{2+} and hence contraction and migration of HASMC, further strengthening the potential role of IL-8 in epithelial-mesenchymal communication and remodelling. However, it was not firmly established whether the in vivo levels of IL-8 seen in bronchial epithelial cells (BEC) would be sufficient to have a significant effect on ASM. In 2012 *Kuo et al* demonstrated that nonphenol not only induces bronchial epithelial apoptosis via the Fas-mediated pathway but also stimulates the bronchial epithelium to secrete IL-6 and IL-8, which cause bronchial smooth muscle proliferation and migration; major features in asthma remodelling (Kuo, Hsu et al. 2012). The observed effect of IL-8 on neutrophils is to activate phospholipase and mobilise intracellular Ca^{2+} which has led to speculation that a similar effect on ASMC would be to cause contraction giving IL-8 a potential role in causing abnormal airway structure in asthma (Govindaraju, Michoud et al. 2006).

Marini et al demonstrated increased IL-8 and IL-8 mRNA levels in BEC isolated from asthmatic vs control subjects (Marini, Vittori et al. 1992). In vitro studies by *Sur et al* showed increased IL-8 in sputum from subjects with moderate to severe asthma compared to those with mild asthma (Sur, Ying et al. 2012). Others have postulated that raised IL-8 levels in serum and sputum area associated with systemic inflammation and loss of asthma control (Silvestri, Bontempelli et al. 2006, Maneechotesuwan, Essilfie-Quaye et al. 2007).

1.4 AIRWAY REMODELLING AND BRONCHIAL HYPER-RESPONSIVENESS

Although asthma is most commonly described as an inflammatory disease of the conducting airways with epithelial dysfunction; airway remodelling (alteration in structural cells and tissues leading to persistent airway-wall thickening) was first described 90 years ago by Huber and Koessler in their description of fatal asthma (HL 1922). Remodelling is a modification in the normal structure and organisation of tissues and usually occurs in response to a stressor. It has been described in most organs including skin (Jorgensen 2003), blood vessels (Rizzoni, Muiesan et al. 2009), heart (Minicucci, Azevedo et al. 2009), gastrointestinal tract (Lawrance, Maxwell et al. 2001) and respiratory system. Airway remodelling in itself is not unique to asthma; it has been described in other pulmonary diseases such as COPD (Chanez, Vignola et al. 1997).

While a natural assumption might be that remodelling of the airways occurs as a result of persistent inflammation and smooth muscle contraction associated with persistent and/or severe asthma this has long proven not to be the case. Remodelling has been described in young children (Cutz, Levison et al. 1978), patients with mild intermittent disease (Jeffery, Wardlaw et al. 1989, Roche, Beasley et al. 1989) and in occupational asthma soon after exposure to the sensitising agent (Saetta, Distefano et al. 1992). Basement membrane thickening has been described in very young children, rhinitis patients and even in patients with atopy, prior to the emergence of symptomatic asthma (Djukanovic, Lai et al. 1992, Chakir, Laviolette et al. 1996, Warner, Pohunek et al. 2000, Pohunek, Warner et al. 2005). Therefore, although clinically diagnosed 'irreversible' airway obstruction is demonstrated in persistent severe asthma, it is likely that remodelling occurs very early in the disease and significant airway function is lost at this time (Kelly, Hudson et al. 1988, Ulrik and Lange 1994, Orsida, Li et al. 1999).

Airway remodelling can be divided into changes involving:

1) Epithelial metaplasia and increased epithelial thickness and goblet cell hyperplasia (see also Epithelium section)
2) Deposition of abnormal extracellular matrix (ECM) components in the basement membrane and deeper submucosa,
3) Increased airway smooth muscle
4) Angiogenesis

1.4.1 Epithelial Abnormalities in Remodelling

Many of the epithelial abnormalities seen in asthmatic subjects have already been described (see Epithelium section). In addition to these, and specifically contributing to airway narrowing, is the abnormality of mucous production. The asthmatic epithelium has mucous glands extending to the peripheral bronchioles where in normal subjects they are confined to the cartilaginous airways (Dunnill, Massarella et al. 1969). There is also an increase in the number of mucous-containing goblet cells (Ordonez, Khashayar et al. 2001) and significant hypertrophy of the submucosal gland mass (Carroll, Mutavdzic et al. 2002) in asthmatic subjects with bronchial gland duct ectasia (dilation of the secretory ducts) (Cluroe, Holloway et al. 1989). These changes lead to excess mucous production and contribute to airway wall thickening and luminal narrowing (Aikawa, Shimura et al. 1992, Shimura, Andoh et al. 1996). Increased mucous viscosity can also cause impedance of mucociliary clearance (Pavia, Bateman et al. 1985) and thus render bronchodilators less effective (Houston, De Navasquez et al. 1953). Mucous plugging can occur from second generation airways to the bronchioles and most cases of fatal asthma describe excessive mucous plugging; some cases report up to a 50% occlusion of airway lumen (Saetta, Distefano et al. 1991).

Mucin glycoproteins are the main macromolecular components of mucous and can be expressed as membrane-tethered mucins or secreted mucin. One of the major gel-forming mucins (MUC5AC) has been shown to be present in large amounts in the airways of fatal

asthma cases (Groneberg, Eynott et al. 2002) Other studies have demonstrated an increase in MUC2 and MUC4 mRNA in asthmatic biopsy samples (Fahy 2002).

1.4.2 Subepithelial Fibrosis

1.4.2.1 Lamina reticularis fibrosis

One of the best-recognised features of airway remodelling in asthmatics is fibrosis in the airway wall, at the level of the basement membrane. The basement membrane of lung tissue comprises the basal lamina ('true' basement membrane) and lamina reticularis; they separate the epithelium from underlying mesenchymal layers. The basal lamina comprises Collagen IV, fibronectin and elastin (Merker 1994) whereas the ECM proteins deposited in the lamina reticularis comprise collagens I, III and V and tenascin and to a lesser extent fibronectin but not Collagen VII (Roche, Beasley et al. 1989, Altraja, Laitinen et al. 1996). The basal lamina is of normal thickness in subjects with asthma whereas the lamina reticularis has been shown to be increased in thickness early in the disease. In subjects without asthma the lamina reticularis is approximately 2-3µm in thickness whereas in asthmatic subjects this is increased two-three fold (Roche, Beasley et al. 1989, Brewster, Howarth et al. 1990). This process contributes to airway wall thickening and subsequent reduced airway distensibility and increased airflow limitation (Ward, Johns et al. 2001). This is thought to be a unique feature of asthma (Roche, Beasley et al. 1989) and can be used to differentiate between asthma and COPD. The lamina reticularis can, in fact, be disturbed in COPD but it looks fragmented and non-homogenous and is not markedly thicker compared with the compact homogenous basement membrane thickening demonstrated in asthma (Kosciuch, Krenke et al. 2012, Soltani, Muller et al. 2012). Detailed imaging of the lamina reticularis of asthmatic subjects shows that the collagen fibrils that are laid down by this myofibroblast layer are structurally different to those seen in normal airways and arranged in a way that suggests a different matrix structure (Saglani, Molyneux et al. 2006).

1.4.2.2 Submucosal extracellular matrix

The ECM is a complex network of macromolecules that fills the extracellular space of the airway wall. Its components are secreted by airway cells and comprise fibrous proteins

(collagen, elastin) and structural or adhesive proteins (fibronectin, tenascin) embedded in a polysaccharide gel. Originally thought to be an inert scaffolding present to support the airway wall and maintain the structure of surrounding tissues, it is now known that the ECM can itself influence cell functions such as development, migration and proliferation (Mcgowan 1992, Raghow 1994).

The composition of the extracellular matrix is dynamic and its synthesis and degradation controlled by fibroblasts, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). This process can become deregulated by aspects of the microenvironment (such as inflammation) and if the balance shifts towards increased matrix deposition, subepithelial fibrosis can occur.

Asthmatic subjects have been demonstrated to have an altered profile of ECM proteins. Collagens I, III and V have been shown to be increased in the submucosa of asthmatic subjects and the orientation and fibre thickness of collagen I fibres appear to be different to that found in healthy controls. It is thought that these changes contribute to the airway wall 'stiffness' seen in remodelling (Wilson and Li 1997, Hoshino, Nakamura et al. 1998, Chakir, Shannon et al. 2003, Johnson, Burgess et al. 2004). In vivo collagen is synthesized in the form of procollagen α-chains R6 which assemble in units of 3 into a procollagen molecule inside fibroblasts. Each chain has a C-terminal extension peptide that aids triple-helix formation (L 1980). Soluble procollagen releases the C-terminal peptide from its C-terminus and is then deposited in tissues as insoluble collagen (L 1980). Asthmatic subjects show the same levels of pro-collagen as healthy controls so it is likely that the increase in ECM collagen is determined by increased conversion of procollagen to collagen (Dube, Chakir et al. 1998). Proteoglycans also have an important function in the ECM, they are able to interact with cytokines and growth factors in order to influence protein storage within the matrix. Several proteoglycans, such as tenascin (Laitinen, Altraja et al. 1997), versican (Huang, Olivenstein et al. 1999) and laminins β_2 and α1 (Altraja, Laitinen et al. 1996) have been found to be increased in the submucosa asthmatic subjects and a study by *Westergen-Thorssen et al* demonstrated that those with the highest levels of BHR had a 4-fold increase in the amount of total proteoglycans (Westergren-Thorsson, Chakir et al. 2002). Asthmatics also demonstrate altered elastin staining, a vital component in maintaining bronchial patency and a contributor to airway recoil (Bousquet, Lacoste et al. 1996). In addition, the enzyme responsible for elastin degradation (elastase) is increased in sputum from

asthmatic subjects (Vignola, Bonanno et al. 1998), suggesting that abnormally increased degradation may be responsible for the lower levels of elastin than in healthy controls.

1.4.2.3 Deposition and degradation of ECM proteins

ECM deposition in airway tissues is dependent on the balance between production and degradation of fibrillar proteins. Biopsy specimens from asthmatic airways show significantly increased numbers of fibroblasts in the submucosa compared with normal subjects (Brewster, Howarth et al. 1990) and this has been correlated with increased RBM thickness (Hoshino, Nakamura et al. 1998). It is thought that collagen deposition is controlled by fibroblast cells. Fibroblasts are large, flat stellate cells with long cytoplasmic extensions that are closely associated with the basal epithelium (Brewster, Howarth et al. 1990). They are the main structural cells of the mesenchymal layer and are thought to be ideally situated to mediate the events of fibrosis and remodelling that occur in subepithelial layers. Under the influence of epithelial damage and cytokines such as TGF-β_1, fibroblasts can differentiate into a bioactive myofibroblast form; a number of studies have shown an increase in myofibroblasts in the submucosal region in asthmatic airways compared to control (Brewster, Howarth et al. 1990, Benayoun, Druilhe et al. 2003). Myofibroblasts secrete inflammatory mediators and extracellular matrix components such as tenascin C, fibronectin, lumican, biglycan and collagens I, III and V. (Karjalainen, Lindqvist et al. 2003) In addition to the secretion of collagen, myofibroblasts also produce more TGFβ_1 (Boxall, Holgate et al. 2006), further prolonging the fibrotic process.

Matrix metalloproteinases (MMPs) are responsible for the degradation of ECM proteins such as collagens, fibronectin, laminin, proteoglycans and elastin. They can also influence the behaviour of the ECM by the release of certain growth factors and other bioactive molecules which themselves have an impact on ECM behaviour. Collagenases (MMP-1 and MMP-13) degrade interstitial collagens, gelatinases (MMP-2 and MMP-9) degrade BM components such as fibronectin and elastin) and the stromolysins (MMP-3, MMP-10 and MMP-11) target a wide number of ECM proteins. Several types of MMPs have been shown to be reduced in the submucosa of samples from biopsies from asthmatic subjects, suggesting another pathway by which the balance of proteins in the ECM could become deranged. MMP-3 is produced at lower levels by fibroblasts from asthmatic subjects than

controls (Tremblay, Chakir et al. 1998); as is MMP-2 (gelatinase A)(Laliberte, Rouabhia et al. 2001). The fibroblasts themselves are also less effective in the degradation of collagen (Laliberte, Rouabhia et al. 2001).

Tissue-specific inhibitors of metalloproteinases control the action of MMPs by binding to them and inhibiting their activation. It has been proposed that an increased level of TIMPs in asthmatics would lead to MMP inhibition and thus reduced degradation of ECM proteins (Laliberte, Rouabhia et al. 2001). MMP-9 is likely to be an important MMP in asthma (Lee, Lee et al. 2001, Ohbayashi and Shimokata 2005); it is found in inflammatory cells (Dahlen, Shute et al. 1999), bronchial epithelium and submucosa (Han, Junxu et al. 2003). In healthy states it should be balanced with TIMP-1 in a 1:1 ration to maintain a balance of ECM protein production and degradation. Several studies have demonstrated an imbalance in this profile in those with asthma. *Mautino et al* showed that in chronic asthma TIMP is increased in proportion to MMP-9 (Mautino, Henriquet et al. 1999) and this correlates with airway wall thickening (Vignola, Paganin et al. 2004, Matsumoto, Niimi et al. 2005). *Ohno et al* demonstrated increased TIMP-1 mRNA and protein levels in asthmatic alveolar macrophages taken from BAL sample (Ohno, Ohtani et al. 1997); *Cataldo et al* demonstrated similar findings in induced sputum (Cataldo, Gueders et al. 2004).

Factors such as TGFβ and Relaxin are molecules that can influence the balance of MMP and TIMP in lung tissue and therefore are implicated in the process of remodelling (see 1.4.7 Other Factors Involved in Remodelling).

1.4.3 Airway Smooth Muscle

An increase in airway smooth muscle is one of the best documented changes in asthma and considered to be one of the major components of airway obstruction. There are reports of increase in smooth muscle cell size (hypertrophy) (Woodruff, Dolganov et al. 2004), smooth muscle proliferation (hyperplasia) (Benayoun, Druilhe et al. 2003) and, more recently, migration of ASM cells towards the epithelium as a contributor to increased smooth muscle mass (Madison 2003). Not only do they contribute to remodelling but also participate in

the propagation of the inflammatory and remodelling process by their ability to release pro-inflammatory cytokines, chemokines and ECM proteins.

Smooth muscle cells interact with the chronic inflammatory process seen in asthmatic airways via cellular adhesion molecules, cytokine receptors and Toll-like receptors expressed on their surface (Joubert and Hamid 2005, Joubert, Lajoie-Kadoch et al. 2005). Studies have shown that $CD4^+$ T cells are able to drive ASM proliferation in vivo (Ramos-Barbon, Presley et al. 2005) and that intracellular adhesion molecule 1 (ICAM-1) and VCAM-1 mediate a pathway whereby activated T lymphocytes are able to adhere to ASM cells and stimulate DNA production (Panettieri, Murray et al. 1995). Other cells such as mast cells, eosinophils and neutrophils have been shown to interact with ASM via ICAM-1 and VCAM-1 thus leading to the supposition that interactions of ASM with inflammatory cells contribute to tissue remodelling (Hughes, Arthur et al. 2000, Brightling, Bradding et al. 2002, Bhandari, Choo-Wing et al. 2006).

1.4.4 Angiogenesis

In lung tissue there is an extensive network of systemic capillaries running beneath the surface from central airways to peripheral bronchioles. At a deeper level they converge to form sinuses which anastomose with the pulmonary capillaries that are drained by pulmonary veins. These blood vessels are an important source of inflammatory cells and act as mediators of the inflammatory cascade. There is evidence to suggest that new vessels form in states of chronic inflammation (angiogenesis) mainly in the area below the basal lamina (Battegay 1995). These new vessels show increased levels of permeability and oedema.

In asthma several abnormalities of the microvasculature have been described. Bronchial biopsies from patients with even mild asthma have increased vascularity and increased blood flow compared with those taken from controls; the vessels are also larger in size (Li and Wilson 1997). In addition it would appear that the vessels from asthmatic subjects may be structurally different as endothelial gaps in the bronchial mucosal microvasculature have been detected in biopsies taken from asthmatic subjects (Nauck, Roth et al. 1997).

This leads to increased oedema in the airway wall which has been shown to contribute to airway wall thickening and therefore remodelling (James, Pare et al. 1989).

Vascular endothelial growth factor (VEGF) controls physiological and pathophysiological angiogenesis. It is synthesized by alveolar epithelial cells, bronchial epithelial cells, smooth muscle cells, fibroblasts and alveolar macrophages (Mura, dos Santos et al. 2004) and its contribution to the pathogenic angiogenesis that takes place in asthma has been recognized. *Ghelfi et al* demonstrated that the overexpression of VEGF in transgenic mice induced leucocyte infiltration, increased Il-13, collagen deposition and smooth muscle hyperplasia with significant angiogenesis, airway wall oedema and vascular remodelling (Ghelfi, Yu et al. 2013). They also demonstrated an amplified T_H2-mediated response following allergen challenge.

Other molecules thought to be of importance in asthmatic angiogenesis are pro-angiogenic mediators angiopoeitin (Ang)-1 and -2, via their effects on VEGF regulation. Ang-1 can induce migration and growth of endothelial cells, it also promotes vessel integrity. When Ang-1 and VEGF are co-expressed Ang-1 is able to promote an increase in capillary diameter and stimulate formation of new blood vessels therefore angiogenesis is enhanced (Thurston, Suri et al. 1999). Ang-2 is mainly expressed in areas of chronic inflammation and is a mediator of epithelial necrosis. It is thought that I VEGF, ang-1 and ang-2 play coordinated roles in airway angiogenesis and vascular remodelling and could potentially be a target for inhibiting angiogenesis in asthma.

1.4.5 Bronchial Hyper-responsiveness and Remodelling

Bronchial hyper-responsiveness is one of the distinguishing features of asthma but is still not fully understood, neither is its interaction with airway remodelling and inflammation. BHR has been shown to correlate with some markers of inflammation (Dupont, Rochette et al. 1998) and improve with anti-inflammatory treatment (Booth, Richmond et al. 1995, Pavord, Jeffery et al. 2009) therefore it was supposed that remodelling was a consequence of inflammation. However remodelling can be present in young children whose airways have had little time to be significantly exposed to inflammation and recent studies have

demonstrated that repeated bronchoconstriction without additional inflammation leads to airway remodelling (Grainge, Lau et al. 2011).

The clinical consequence of severe airway remodelling is fixed airway obstruction and several studies have demonstrated a correlation between subepithelial fibrosis and BHR (Chetta, Foresi et al. 1997, Hoshino, Nakamura et al. 1998, Hoshino, Takahashi et al. 1999) or FEV_1 but this has not been reported in all cases (Brewster, Howarth et al. 1990). The traditional supposition was that any amount of remodelling would be deleterious and that an airway that had undergone significant 'remodelling' would be altered geometrically (increased thickness) and mechanically (increased stiffness) therefore limiting deep inspirations and increasing smooth muscle contractility i.e. allowing the airways to narrow more in response to stimuli compared with those of a non-asthmatic subject (Lambert, Wiggs et al. 1993, Boulet, Laviolette et al. 1997, Oliver, Fabry et al. 2007). This has been countered with more recent studies which conversely suggest the opposite; that remodelling is, in fact, a mechanism designed to protect against dramatic and potentially harmful BHR and reduce airway compressibility (Brackel, Pedersen et al. 2000, McParland, Macklem et al. 2003, Niimi, Matsumoto et al. 2003).

1.4.6 Remodelling and the EMTU

Animal studies show that a chronically injured epithelium can produce TGF-β, EGF, fibroblast growth factors, platelet derived growth factors (PDGFs), insulin-like growth factors (IGFs), neurotrophins and vascular endothelial growth factors (Leung, Niimi et al. 2006, Locke, Royce et al. 2007). This effect can be stimulated by mechanical stress, viral infection, chemical injury or exposure to inflammatory cells such as eosinophils and neutrophils. One of the predominant effects of all of these factors is to cause fibroblast proliferation or stimulate their transformation to myofibroblasts (Choe, Sporn et al. 2003). *Plopper et al* demonstrated that, in primates, air pollutants and allergens interacted with airway epithelial cells in early life leading to BHR, T_H2 inflammatory response and airway remodelling (figure 4). These changes persisted even when the stimulus was removed (Joad, Kott et al. 2006, Plopper, Smiley-Jewell et al. 2007).

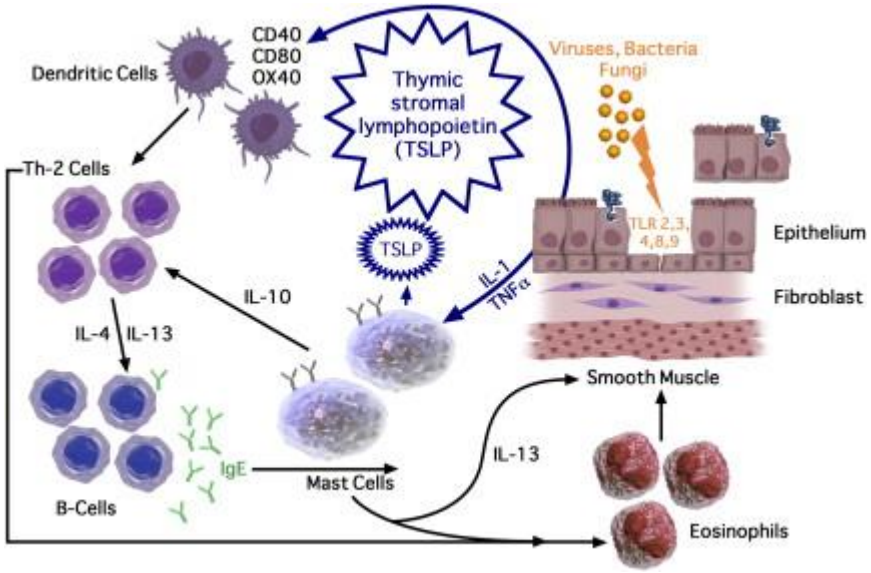


Figure 4: Schematic representation of the interrelationships between epithelial injury and aberrant repair and chronic inflammation in moderate-severe asthma. Immune and inflammatory cells interact with structural cells both to maintain the inflammatory response and to drive airway wall remodeling.

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1.4.7 Other Factors Involved in Remodelling

1.4.7.1 Transforming growth factor-β (TGF- β)

TGF-β is a pleiotropic cytokine which has several different functions and can be produced from several sources, although in asthmatic patients the main source appears to be eosinophils (Minshall, Leung et al. 1997, Redington, Madden et al. 1997). It has been demonstrated to have a significant effect in increasing airway smooth muscle and epithelial cell apoptosis and for this reason it has been strongly implicated in the process of airway remodelling. TGF-β promotes differentiation of fibroblasts to myofibroblasts (Michalik, Pierzchalska et al. 2009) and induces MMPs and TMPs thus influencing extracellular matrix turnover (Mattos, Lim et al. 2002). It also enhances proliferation of ASM cells and contributes to the migration of ASM towards the epithelium (Chen and Khalil 2006, Ito, Fixman et al. 2009).

TGF-β is secreted from cells in association with its propeptide, in an inactive state; therefore activation of this molecule is critical to influencing disease states. Several studies have described increased levels in asthma and its activation has been explained by several mechanisms (Sagara, Okada et al. 2002, Torrego, Hew et al. 2007). Epithelial cells are believed to activate TGF-β when damaged, mast cells are thought to release proteases from their granules which have a role in TGF-β activation (Lindstedt, Wang et al. 2001, Howat, Holgate et al. 2002, Tatler, Porte et al. 2008, Woodman, Siddiqui et al. 2008) and recently integrins have been the source of much interest. Integrins are cell surface molecules involved in cell-cell and cell-matric interactions. Four of the twenty-four described integrins have been reported to activate TGF-β, the best characterised are the αvβ6 (Munger, Huang et al. 1999) and αvβ8 (Mu, Cambier et al. 2002). Deletion of fibroblast-specific αvβ8 has been demonstrated to reduce airway remodelling by reducing dendritic cell migration (Kitamura, Cambier et al. 2011). In 2001 *Tatler et al* described that bronchospasm inducing agents could activate TGF-β in human ASM cells and that ASM cells from asthmatic subjects produced more TGF-β than those from non-asthmatic subjects (Tatler, John et al. 2011). They identified that this process took place via the αvβ5 integrin.

1.4.7.2 Periostin

Periostin is one of the most highly differentially expressed genes in asthma (Woodruff, Boushey et al. 2007). It is a 90-kDa disulphide linked matricellular protein belonging to the fasciclin family. It is expressed in connective tissues such as periodontal ligament, heart valves, myocardium, tendons, skin and bone (Kii, Amizuka et al. 2006, Kikuchi, Kashima et al. 2008). It reacts with integrins (αVβ3, αVβ5 and α6β4) to control effects of cell proliferation, cell migration, epithelial to mesenchymal transformation and alteration of connective tissue properties. It plays a significant role in modulation of cardiac myocytes, inducing healing after cardiac damage; it has also been the source of considerable interest in cutaneous wound repair (Gillan, Matei et al. 2002, Butcher, Norris et al. 2007, Dorn 2007).

Periostin has been shown to be overexpressed in the subepithelial region in asthmatic bronchial epithelial cells (Takayama, Arima et al. 2006) and is thought to play a part in the subepithelial fibrosis of asthma via its effects on collagen in the extracellular matrix of the bronchial lamina reticularis. *Norris et al* have demonstrated that periostin binds type 1

collagen to promotes fibrillogenesis via activation of lysyl oxidase, a catalytic enzyme involved in cross-linking of collagen (Norris, Damon et al. 2007).

Because the effects of periostin are primarily in the subepithelial layers most studies have studied Periostin derived from fibroblasts; however *Sidhu et al* demonstrated that Periostin is upregulated in asthmatic bronchial epithelium in response to IL-13 and secreted in a basal direction (Sidhu, Yuan et al. 2010). In addition, medium from epithelial cells over-expressing periostin caused TGF-β-dependent secretion of type 1 collagen by airway fibroblasts. These findings have led to the suggestion that periostin may be a key factor in epithelial-mesenchymal communication to influence fibrotic events in the subepithelial layer in asthma.

Peripheral blood (serum) levels of periostin are significantly raised in patients with asthma and seems to be a more reliable indicator of airway eosinophilic inflammation than blood eosinophil levels or fractional exhaled nitric oxide (FENO) (Jia, Erickson et al. 2012). It has been associated with a higher rate of FEV_1 decline in asthmatic patients on ICS (Kanemitsu, Matsumoto et al. 2013) and it has also been suggested that it may be associated with a more 'unstable' form of asthma i.e. those with latent inflammation (Kato, Takahashi et al. 2013).

It is currently used within the context of clinical trials (as a surrogate marker for T_H2 inflammation) by identifying patients in whom novel asthma therapies may be helpful. Novel agents such as anti IL-13 therapy, Lebrikizumab, showed that having high Periostin levels predicted a better response to therapy (in post-hoc analysis) (Corren, Lemanske et al. 2011). Periostin may prove to be helpful in developing treatments for refractory eosinophilic asthma in the future.

1.4.8 Viral Infection and Remodelling

Viral infection with human rhinoviruses is a major cause of exacerbation in patients with asthma, both adults and children (Kotaniemi-Syrjanen, Vainionpaa et al. 2003, Miller, Edwards et al. 2009, Miller 2010). Infection leads to amplification of the inflammatory cascade with release of pro-inflammatory mediators such as cytokines, chemokines,

interferons and growth factors (Gern and Busse 2002, Jackson, Gangnon et al. 2008). Recent studies have shown that epithelial cells infected with rhinovirus express markers strongly associated with remodelling such as amphiregulin (an epidermal growth factor that alters the repair process) and vascular endothelial growth factor (a proangiogenic activator) and activin A (a member of the TGF-β family) (Jackson, Gangnon et al. 2008, Leigh, Oyelusi et al. 2008). Rhinovirus has also been detected in the subepithelial layers, including fibroblasts, in asthmatic subjects. Recent developments in this field have been able to demonstrate that fibroblasts from the airways of asthmatic subjects enhance the replication of rhinovirus and lead to increased production of IL-6 and IL-8 (Bedke, Haitchi et al. 2009); this response was augmented in TGF-β-treated fibroblasts. The relationship between infecting viruses and the release of substances implicated in structural alteration; combined with the presence of viruses in subepithelial structures suggest that the viruses may be linked to remodelling (Hewson, Jardine et al. 2005, Wark, Johnston et al. 2005, Message, Laza-Stanca et al. 2008).

1.5 THE ISLE OF WIGHT 1989 BIRTH COHORT

An unselected whole population birth cohort was established on the Isle of Wight, UK, in 1989 to prospectively study the natural history of allergies from birth to age 18 years. The island is close to the British mainland, semi-rural, with no heavy industry; the population is 99% Caucasian. The local Research Ethics Committee approved the study (06/Q1701/34). Of the 1536 children born between 1 January, 1989, and 28 February, 1990, informed consent was obtained from parents to enrol 1456 newborns. Children were followed up and characterised for asthma and allergic disease at the ages of 1 (n = 1167), 2 (n = 1174), 4 (n = 1218), 10 (n = 1373) and 18 years (n = 1313) (Arshad, Stevens et al. 1993, Kurukulaaratchy, Waterhouse et al. 2005). Novel longitudinal information on positive and negative transition of eczema and rhinitis throughout childhood and adolescence has been published as a result (Ziyab, Raza et al. 2010, Kurukulaaratchy, Karmaus et al. 2011).

At 18 years the participants were characterised into 4 groups: persistent asthma with BHR (having asthma at ages 10 and 18 years), asthma remission (having asthma at age 10, but no evidence of asthma at 18 years i.e. no symptoms or treatment used for at least 12 months), new onset asthma (asthma presence at age 18 years but not before) and never asthma.

We assessed dynamic changes in asthma status over the first 18-years of life with particular regard to sex and atopy. Asthma was defined at both 10 and 18 years as “ever asthma” and either “wheezing or whistling in the chest in the last 12 months “or “current treatment for asthma”. Contrary to many prior studies, a significant rise occurred in overall asthma prevalence from 10- to 18-years; from 14.7% to 17.9% (p = 0.027). At 10-years there was a significant male predominance for asthma (17% vs 12.4%, p = 0.018) whereas at 18-years there was a non-significant female predominance (19.4% vs 16.4%, p = 0.18).

Superficially that might be interpreted as supporting the notion of an adolescent “sex switch” in asthma prevalence. However on stratified analysis, it was mainly atopic asthma that increased between the ages of 10-and 18-years from 9.7% to 13.4% (p = 0.014) while non-atopic asthma did not change significantly during this period. Furthermore, the increase in atopic asthma was only statistically significant in females (p = 0.021). Females also showed non-significant trends for developing adolescent non-atopic asthma (5.8% to

7.9%) while males showed a trend for growing out of non-atopic asthma. In summary, stratified transitional analysis in the Isle of Wight Birth cohort demonstrated significant and previously undocumented rise in the prevalence of asthma during adolescence due to lower rates of remission than have been previously described. The prevalence of asthma at 10 years was significantly higher in males than females; however at 18 years there was no significant difference between the sexes. When stratified by atopic status it could be seen that there was a significant increase in atopic asthma in females during adolescence. This is contrary to the traditional expectation of a rise in adolescent female asthma status through an increase in non-atopic disease. The prevalence of asthma in males during adolescence showed no significant change due to relatively equal proportions developing atopic asthma and losing non-atopic asthma (Figure x).

At 18 years asthma prevalence rose to 17.9% from 14.7% at 10 years; of those with 10 year data 63.1% had persistent asthma and 28.3% had adolescent-onset asthma. There was a non-significant trend to female preponderance; in comparison with other studies mentioned previously which have shown a statistically significant difference between the sexes (Nicolai, Pereszlenyiova-Bliznakova et al. 2003, Mandhane, Greene et al. 2005, Tollefsen, Langhammer et al. 2007). Disease severity was found to be comparable in the adolescent-onset and persistent asthma groups; however self-rated health status was significantly higher in adolescent-onset group (i.e. more healthy) and prescription of anti-asthma treatment was higher in this group.

Due to the differences in adolescent growth patterns between the sexes, lung function was analysed by gender. In males, those with childhood-onset asthma had significantly lower FEV_1 and FEF_{25-75} than never asthmatics age 10 years, but if they grew out of asthma during adolescence, by the age of 18 years these differences had disappeared i.e. their lung volumes had 'caught up' with those who had never had asthma. If their asthma persisted through adolescence their lung function remained significantly lower. In females, those who developed asthma during adolescence demonstrated lower gain in FEV_1 between the age of 10- and 18 years, resulting in them having significantly lower FEV_1 by the age of 18 years.

CHAPTER 2 – MATERIALS, METHODS AND ANALYSIS

2.1 THE STUDY

2.1.1. The Hypothesis

In subjects with both clinical remission of asthma and absence of BHR, there is evidence of airway inflammation, activation of the EMTU and changes associated with remodelling.

2.1.2 The Aims of the Study

This was a nested case control study. To test the hypothesis:

1. Three groups (n = 15 in each group) of subjects will be selected who have previously participated in the IoW birth cohort, depending on the presence of asthma at age 10 and 18 years. These include; persistent asthma (PA), asthma remission (AR) and a control group of never asthma (NA).

2. These 45 subjects will be further characterised in each group for lung function, bronchial responsiveness and atopy.

3. A bronchoscopy will be performed on each participant and the bronchial biopsies and brushings analysed for evidence of activation of the EMTU and tissue remodelling.

4. Information derived in objectives 1 and 2 will be used to determine whether those with clinical remission of asthma and no evidence of BHR have evidence of ongoing airway inflammation and/or remodelling.

5. To identify novel markers associated with airway remodelling in these groups.

2.1.3 Ethics and Research and Development

Ethical approval was granted by the National Research Ethics Service for South Central and Portsmouth (Ref: 10/H0501/66). A substantial amendment application was granted on 25th of May 2011.

The Research and Development Department at the Biomedical Research Unit, Southampton General NHS Trust granted approval on 28th April 2011 (Ref: RHM MED 0956).

The Research and Development Department at St Mary's Hospital, Isle of Wight Primary Care Trust granted approval on 13th July 2011 (CSP Ref 59740).

2.1.4 Power Calculation

A post-hoc power calculation was performed for the above tests showing statistical significance based on suggestions in the literature for non-parametric tests (Lehmann 1998, Rosner and Glynn 2011). We used Wilcoxon rank sum tests for the power calculations. Multiple testing is adjusted within each measure (3 tests for each measure) and the adjusted significance level is 0.017 based on the Bonferroni approach.

Table 1a Power calculations based on non-parametric Wilcoxon rank sum test.

Test	Collagen I		Collagen III		CBT	
	Test statistics	**Power**	**Test statistics**	**Power**	**Test statistics**	**Power**
PA vs AR	0.34 (n1=12, n2=14)	33.7%	0.21 (n1=12, n2=14)	52.4%	0.34 (n1=9, n2=12)	30.8%
PA vs NA	0.81 (n1=12, n2=13)	56.1%	0.93 (n1=12, n2=14)	75.0%	0.99 (n1=9, n2=9)	72.5%
AR vs NA	0.60 (n1=9, n2=9)	37.8%	0.70 (n1=14, n2=14)	45.2%	0.99 (n1=12, n2=9)	76.9%

Since t-tests are robust to non-normality, in addition, we estimated power based on two sample t-tests. Multiple testing was adjusted as above (adjusted significance level 0.017).

Table 1b lists the power of each test. Power estimates based on the two-sample tests are higher compared to those based on non-parametric approach, which is as expected.

Table 1b Power calculations based on two sample t-tests.

Test	Collagen I		Collagen III		CBT	
	Difference (%) (SD)	**Power**	**Difference (%) (SD)**	**Power**	**Difference (%) (SD)**	**Power**
PA vs AR	7 (6)	65.6%	6 (7)	36.8%	2 (2)	36.8%
PA vs NA	12 (6)	99.1%	11 (7)	90.2%	5 (2)	99.2%
AR vs NA	5 (6)	38.1%	6 (7)	38.7%	4 (2)	95.5%

Combining the calculated power based on two methods, even with the small samples in each of the three groups, the power is acceptable and ranged from ~30% to ~77% based on non-parametric tests, and ~40% to >99% for parametric test after adjusting for multiple testing for each marker (3 tests per marker).

The power calculations, as described above, were based on analysis of collagens I and III and CBT. Some of the other indices had smaller numbers of samples and may not have had sufficient power to detect differences between the groups. This may have led to a type 2 statistical error i.e. a false negative when analysing those indices.

This section was written with the assistance of Hongmei Zhang).

2.1.5 Study Personnel and Recruitment

All 1456 children originally recruited to the 1989 Isle of Wight Birth Cohort Study were considered for entry into the EMTU study. Written information in the form of a Patient Information sheet (PIS) (v3_27thApril2011) (see **Appendix 2**: **Supplementary Materials**) and an invitation letter with tear-off reply slip and pre-paid return envelope were sent in batches of approximately 50 to participants in each group. In the following weeks they were contacted by telephone (by SE or FM) to briefly discuss the study and invited for a screening visit. They were invited to attend the Isle of Wight David Hide Asthma and Allergy Centre for the first visit and informed written and verbal consent was obtained prior to participation.

In order to recruit the required number of participants with persistent asthma, in 04/2012 further approval from the Research Ethics Council was sought to recruit from the smaller 1990 Intervention Study cohort. This was a randomised controlled trial with intervention (allergen avoidance) administered to one group. Only those with persistent asthma who had been part of the control group were included. Approval from the Research Ethics Council was obtained on 15/05/2012 (10/H0501/66) and suitable participants were identified by the David Hide Asthma and Allergy Centre Manager (SM).

The Research Fellow (SE) was responsible for co-ordination of the study including obtaining approvals from ethic committees and the Research and Development Departments at St Mary's Hospital, Isle of Wight and Southampton General Hospital, developing the Case Report Forms and liaising with GPs to let them know that their patient was participating in the study and the results of any investigations performed if relevant to the participant's health. A study nurse (FM) and managerial staff (SP) performed tasks such as production of recruitment letters, visit reminder letter and other administration tasks. The EMTU study was conducted over a 15-month period between June 2011 and October 2012.

The Research Fellow (SE) and a Research Nurse (FM) were responsible for contacting the participants either by telephone or e-mail to arrange the first visit. For reasons of continuity and consistency the Research Fellow (SE) was responsible for carrying out the first visit interview and procedures as well as arranging the second visit and performing pre-

bronchoscopy assessment, bronchoscopy procedure, post-bronchoscopy check and counselling and follow-up phone call the following day.

2.1.6 Exclusion Criteria

The impact of a bronchial challenge test with mannitol on pregnancy is not well documented therefore we have excluded pregnant women from taking part in the study. Any participant not able to satisfy the criteria for inclusion into *never asthma (NA), persistent asthma (PA)* or *asthma remission* (AR) groups were classified as *intermittent wheezers* and not included in the study.

Patients were well matched for gender and smoking status between the three groups. This was unintentional and, should there have been differences, would have been statistically corrected for in the analysis.

2.2 CLINICAL ASSESSMENT

All information from Visits one and two were recorded on the Case Report Form (CRF) (see **Appendix 2**: **Supplementary Materials**) using additional sheets, if required.

2.2.2 Visit One

The first visit for the EMTU study was conducted at the David Hide Asthma and Allergy Centre at St Mary's Hospital, Newport, Isle of Wight. Participants were seen during a one and a half hour visit to the Research Centre and the visit was conducted by a Research Fellow (SE) under the supervision of the Principal Investigator (SHA) and assisted by a member of nursing staff when required (FM).

For each participant attending the Research Centre, data collection included height, weight, pulse, blood pressure and peripheral oxygen saturations. All participants answered the ISAAC core questionnaire (Asher, Keil et al. 1995) and, where appropriate, an additional questionnaire focusing on allergic symptoms. They underwent skin prick testing to common allergens, baseline lung function at spirometry and bronchial challenge to mannitol. Signs of allergic disease were documented after a brief clinical examination of the respiratory system; skin, ear, nose and throat were examined if thought to be relevant.

Participants were rescheduled if they had a recent chest infection (within last 14 days) or were taking oral steroids. They were asked to refrain from consuming caffeine, undertaking strenuous exercise or smoking on the day of the visit. Female participants were asked if they were pregnant and excluded if they were.

Preparation for the bronchial provocation test required that participants abstain from using their asthma medication in accordance with the table 2a. This table was in the PIS and also in the appointment reminder letter (see **Appendix 2**: **Supplementary Materials**). During the telephone consultation they were given specific instructions tailored to the medication they were using (if any). They were also advised in both the PIS and during the telephone discussion that if they were unable to tolerate stopping the medications or developed asthma symptoms after stopping the medications they should resume their medication as

normal and contact the David Hide Asthma and Allergy Centre (in hours) or the study mobile (out of hours) as soon as possible. Participants who were unable to stop asthma medication as required could be accepted into the study but would not undergo a bronchial provocation test.

Time to withhold	Medication
6-8 hours	Inhaled non-steroidal anti-inflammatory agents
8 hours	Short acting beta-2 agonists
12 hours	Inhaled corticosteroids
12 hours	Ipratropium bromide
24 hours	Long-acting beta-agonists Combination inhalers containing the above
24 hours	Theophylline tablets
72 hours	Tiotropium bromide
72 hours	Anti-histamine tablets
4 days	Leukotriene receptor antagonists

Table 2a: Medications to be stopped (and timescale) before performing the bronchial provocation test.

2.2.2 Questionnaires

Detailed interviewer-administered questionnaires were completed at Visit one regarding asthma and other allergic disease (hayfever and eczema) symptoms. For the sake of consistency the same individual (SE) conducted all questionnaires. All participants completed the ISAAC (International Study of Asthma and Allergies in Childhood) questionnaire which is a standardised research tool and has been used at previous study follow-up visits (see **Appendix 2**: **Supplementary Materials**).

Information was recorded at visit one about socioeconomic factors such as the participants' current employment status, type of housing and total family income. Where the participant still lived in the family home the family income was recorded, where the participant had moved out of the family home their own income and that of any partner was recorded. If

the participant lived in shared housing/University halls their own income was recorded if they were in employment and if they were not in employment no answer was recorded. Information regarding home environmental exposure to pollution, tobacco smoke and pets was recorded. If the participant lived at two residences (e.g. University residence and family home) the exposure was recorded for the home at which they spent the most time.

Information was recorded regarding the amount of time spent using a computer and watch television on weekdays and at the weekend. This was then expressed as *total hours per week* spent on each activity. Pollution exposure was assessed by asking 'How frequently are you annoyed by outdoor air pollution (from traffic, industry etc) in your home if you keep your windows open?' Those answering 'every day' or 'once a week' were classified as *high air pollution exposure* and those answering 'once a month,' 'once a year' or 'never' as *low pollution exposure*. Exposure to traffic was assessed by asking 'How often do cars pass your house on the street less than 100m away' and 'How often do heavy vehicles e.g. trucks and busses pass your house on the street less than 100m away.' Those answering '>10/hour' to either of these were classified as *high traffic exposure* and those answering '1-9 per hour,' '10 per day,' 'seldom' or 'never' were classified as *low traffic exposure*. Exposure to fungal spores was assessed by asking 'Does your home have damp spots on the walls or ceiling?' and 'Does you home have visible moulds or fungus on the walls or ceiling?' Possible exposure to fungal spores was recorded as positive if 'yes' was answered to either of these questions.

Additional information was obtained from those who reported current asthma symptoms using *a supplementary atopic disease questionnaire* again consistent with those used at previous follow-up. This questionnaire obtains information regarding natural history of allergic disease, medication use and asthma severity (see **Appendix 2**: **Supplementary Materials**). BTS criteria were used to classify asthmatic participants into severity categories; this classification is based on asthma medication use and detailed in the table below (table 2b).

The *supplementary atopic disease questionnaire* also elicited eczema and hayfever symptoms. *Current eczema* was defined as answering 'yes' to the question' Have you had an itchy rash coming and going during the last 12 months?' They were also asked whether

this rash had disturbed their sleep at night (never/less than one night a week/more than one night per week). Current hayfever was defined as answering 'yes' to the question 'Have you had a problem with sneezing or a runny or blocked nose when you have not had a cold in the last 12 months?'

Table 2b. BTS asthma severity staging

BTS stage	Description	Criteria
1	Intermittent asthma	Inhaled SABA only
2	Regular preventative therapy	Add ICS 200-800µg/day
3	Initial add-on therapy	Maximise ICS to 800µg/day and add LABA. If LABA not effective stop and add third drug e.g. Montelukast or aminophylline
4	Persistent poor control	Increase ICS to 2000µg/day and add fourth drug e.g. Montelukast, aminophylline or β_2-agonist tablet
5	Continuous or frequent use of oral corticosteroids	Maximal inhaled therapy and add-on therapy exhausted. Regular oral corticosteroids. Refer to a Specialist.

LABA = long-acting β_2 agonist, SABA = short-acting β_2 agonist

2.2.3 Skin Prick Testing

Skin prick testing is a safe, rapid and sensitive method of assessing the presence of allergen specific IgE antibodies in an individual. The procedure was performed by a standardised method identical to that used at the 10 and 18 year follow-ups, to a panel of common aeroallergens and food allergens. Aeroallergens comprised house dust mite (*Dermatophagoides pteronyssinus*), grass pollen mix (timothy grass, rye, meadow, colts foot, june, false out), tree pollen mix (alder, silver birch, hazel), cat and dog epithelia, *Alternaria alternata*, *Cladosporium herbarum*. Food allergens comprised milk, hens' egg, wheat, soya, cod and peanut; histamine dihydrchloride (10mg/ml) and physiological saline acted as the positive and negative controls respectively (Alk-Abello, Horsholm, Denmark).

The participant was required to have avoided antihistamine medication for at least 72 hours prior to the test; if this was not possible they were offered an appointment at a later date or the skin prick test was not performed and the data recorded as missing. Allergens were applied to the volar aspect of the forearm. Single-headed lancets were used and the

skin pricked at an angle of 90°. The weal diameter was recorded at 10 minutes. A skin prick test was defined as positive where the mean weal diameter was at least 3 mm greater than the negative control.

All skin prick tests and their interpretation were performed by the same individual (SE) to maintain consistency. The results were recorded on the CRF (see **Appendix 2: Supplementary Materials**).

2.2.4 Spirometry and BHR Assessment (Mannitol Provocation Test)

Spirometry was performed following American Thoracic Society guidelines to ensure spirometry validity and reproducibility.(Miller, Hankinson et al. 2005) FEV_1, forced vital capacity (FVC) and peak expiratory flow rate were recorded for each participant, all of which were expressed as percent predicted for age, height, sex and ethnic origin. Forced expiratory ratio (FEV_1/FVC) was calculated from the above data. As recommended, the highest of three FEV_1 measurements that were within 5% of each other was recorded as a baseline value. KoKo spirometry software (PDS Instrumentation, Louisville, USA) was used.

Bronchial hyper-responsiveness can be described as the degree of airflow limitation in response to a bronchoconstrictor. It is not exclusive to asthma but is one of the criteria used in its diagnosis (R 1997). Bronchial provocation testing (BPT) uses agents that are grouped into 'direct' and 'indirect' according to the mechanism by which they induce airflow limitation. Direct stimuli e.g. methacholine and histamine induce airflow limitation by direct action on effector cells such as airway smooth muscle cells, bronchial vascular endothelial cells and/or mucous producing cells. Indirect stimuli e.g. mannitol, AMP and hypertonic saline act on intermediary cells such as inflammatory cells, bronchial epithelial cells or neuronal cells, which produce pro-inflammatory mediator or neurotransmitters that interact with the effector cells (Pauwels, Joos et al. 1988, Van Schoor, Joos et al. 2000) (figure 5).

Direct BPTs are very sensitive and a negative test is useful to rule out asthma, however they are not very specific and a positive test can be seen in a number of other clinical conditions such as allergic rhinitis, COPD and infection as well as a proportion of non-asthmatic

subjects (Backer and Ulrik 1992, Kolnaar, Folgering et al. 1997, Brusasco and Crimi 2001). Indirect tests have a higher specificity and correlate better with levels of inflammation and severity of disease (de Meer, Marks et al. 2004, Anderson 2008, Porsbjerg, Brannan et al. 2008).

Mannitol is thought to act through an increased osmolarity in the pericilliary liquid. This results in release of bronchoconstricting mediators from inflammatory cells, which eventually causes smooth muscle contraction (Leuppi, Brannan et al. 2002).

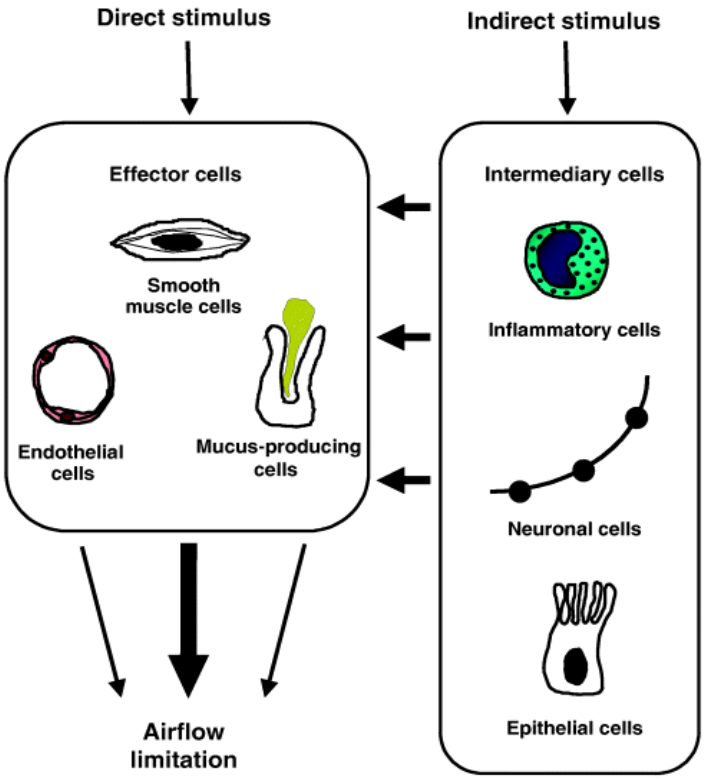

Figure 5: Mechanisms via which direct and indirect stimuli induce airflow limitation.

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Bronchial challenge was performed in this case using mannitol as the stimulant (Anderson, Brannan et al. 1997, Brannan, Porsbjerg et al. 2009, Parkerson and Ledford 2011). A mannitol dry powder inhaler was used which is available as a pre-packaged set in incremental doses, as Osmohale™ (Pharmaxis Pharmaceutical Ltd, Bucks, UK) (table 3). A pre-test spirometry reading was obtained to ensure an FEV_1 of above 70% predicted for age and height. Initial inhalation of 0 mg mannitol (step one) was followed by spirometry

recording to obtain a baseline value. Subsequently, incremental doses (steps 2 through 9) of mannitol were administered as described by the manufacturers (http://www.aridol.info/test-instructions) (figure x). One minute after each dose two forced expiratory manoeuvres were performed and the highest FEV_1 recorded. The provocation dose causing a 15% fall in FEV_1 from the post-0mg value or a 10% fall in FEV_1 from the previous reading was considered to be a positive test and expressed as PD_{15}. If these criteria were not met by the end of the test, after dose 9 (total 635mg mannitol) then it was considered to be a negative test. Those participants who had a positive test or a negative test with symptoms of wheeziness or chest tightness were given two puffs of salbutamol CFC-free (200mcg) via a volumatic spacer. After 10 minutes they were asked to perform a further forced expiratory manoeuvre; their FEV_1 was required to be >95% of the pre-test FEV_1 before they were discharged.

Mannitol inhalators were single use and not used for more than one participant. Any unused capsules from the pack were discarded after the test was complete.

Table 3: Incremental mannitol dosing regime

Step	Mannitol Dose	Cumulative Mannitol Dose
1	0mg	0mg
1 minute break*		
2	5mg	5mg
1 minute break*		
3	10mg	15mg
1 minute break*		
4	20mg	35mg
1 minute break*		
5	40mg	75mg
1 minute break*		
6	80mg	155mg
1 minute break*		
7	160mg	315mg
1 minute break*		
8	160mg	475mg
1 minute break*		
9	160mg	635mg
1 minute break*		

*after each minute break perform two forced expiratory manoeuvres and record the highest FEV_1

The information was recorded on an Excel spreadsheet designed by a member of the research team (SP). The spreadsheet was designed to automatically calculate the percentage drop in

FEV_1 from baseline and percentage drop in FEV_1 from the previous dose (**see Appendix 2: Supplementary Materials**).

The response is expressed as the provoking dose of inhaled mannitol to cause a 15% fall in FEV_1 (PD15). The PD15 is an index of sensitivity that reflects the severity of BHR, therefore the PD15 was further classified into categories of 'severe' (≤35 mg), 'moderate' (≤155 mg) and 'mild' (>155 mg) according to producers information (Brannan, Porsbjerg et al. 2009).

Those with a negative result had the final drop in FEV_1 recorded. If this was lower than the baseline value this was expressed as a total percentage drop in FEV_1.

2.2.5 Urine Testing

A urine sample was collected at visit one from all participants. 2ml of urine was aliquotted into two 1ml Eppendorf tubes for cotinine testing and stored in a -80° freezer.

Female participants were asked for written and verbal consent to perform a pregnancy test using Clearview Easy HCG (human chorionic gonadotrophin) test kit at both visits 1 and 2.

2.2.6 Visit Two

Participants travelled to The Biomedical Research Unit and Wellcome Trust, Southampton General Hospital for the second visit which took approximately 4 hours. They were asked to refrain from consuming food for 4 hours before the procedure and refrain from taking fluids for 2 hours before. They were required to take any inhaled medication as usual before the procedure. Other medication were usually postponed and taken after the procedure. Management of specific medications such as blood-thinners and insulin for diabetes were discussed at the first visit. If a participant developed an illness or sustained an injury that was not fully recovered by the date of the procedure it was cancelled and rebooked at a later date when they were in good health.

Following the procedure they were advised not to drink alcohol, drive a car or use heavy machinery for 24 hours. It was explained to participants that, for reasons of safety, it was

necessary for a chaperone to escort them home after the procedure. This could be a friend, partner or family member of their choosing or a member of the research team (SE or FM). If escorted home by a member of the research team, a member of their family/partner/friend should be at home with them for the rest of the day and overnight. They were given a written information sheet which contained this information and the study mobile number should they need any advice. They were contacted the following day as a follow-up.

2.2.8 Blood sampling

Blood samples were taken at the second visit during cannulation prior to bronchoscopy (to avoid duplicating the procedure.) 20ml of blood was taken and stored as whole blood and serum.

2.2.8 Reversibility

Participants underwent spirometry upon arrival. It is worth noting that the participants with Persistent asthma were taking their inhaled medication as usual. 3 readings were taken of FEV_1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) and the highest of the three recorded in the notes as 'pre-bronchodilator FEV_1'. 2.5mg of Salbutamol was then given through a nebuliser with mouthpiece driven by air. After 15 minutes FEV_1 and FVC were repeated and the highest of the three recorded in the notes as 'post-bronchodilator FEV_1.' If either of the recorded readings were <10% of predicted for sex, age and height a more detailed review took place of the patients respiratory system with possible postponement of the procedure. After the bronchoscopy 3 readings of the FEV_1 and FVC were taken again and if not within 10% of the best pre-procedure reading the participant was given Salbutamol 2.5mg again and underwent medical review.

2.2.9 Bronchoscopy

A fibreoptic bronchoscopy was performed under sedation and local anaesthesia according to the standardized operating policy (SOP) of the Wellcome Trust Clinical Research Facility and as per British Thoracic Society Guidelines([Anon] 2001). Briefly participants received a titrated dose of midazolam (up to a maximum of 5mg) and alfentanyl (up to a maximum of 1000mcg). They received topical lignocaine (10%) spray applied intra-nasally and orally. They were intubated nasally (or orally) and lignocaine applied topically to the vocal cords (2%) and bronchial tree (1%). Maximum total dose of lignocaine applied was 300mg.

A maximum of 6 bronchial brushings and 8 endobronchial biopsies were obtained and following this a 60ml bronchiolar lavage was performed if the participant was tolerating the procedure. Biopsies were processed as follows: 2 for glycol methacylate (GMA), 1 fresh, 1 in RNAlater, 1 snap frozen, 1 for electron microscopy (EM), 2 for whole mount.

2.3 ADVERSE EVENTS

Adverse events were recorded on the CRF and site file. Serious adverse events were reported to the research and development Department according to the Wellcome Trust Adverse Events Reporting Policy. Four adverse events occurred during the study, one serious adverse event and three minor adverse events, they are detailed in table 4.

Table 4. Adverse events	
Type of event	*Outcome*
Adverse event	
1. Participant with asthma developed a chest infection after the procedure	Required a course of antibiotics
2. Participant with asthma had a minor exacerbation of asthma symptoms the day after the procedure	Required a course of oral steroids
3. Participant without asthma had chest discomfort after the procedure and a chest x-ray was performed	Normal x-ray appearance
Serious adverse event	
4. Participant developed headache and vomiting after removal of cannula, unable to keep down fluids	Participant required admission to hospital for observation for 3 hours before being discharged

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 Biopsy Preparation

Two biopsies (if full complement taken) were embedded in GMA and all sections were cut using a microtome to a thickness of 2µm then air-dried for one hour. Each biopsy was sampled twice.

2.4.2 General Methods

Endogenous peroxidase was inhibited by incubating with 0.1% sodium azide and 0.3% hydrogen peroxide for 30 minutes. Blocking culture medium (20% newborn calf serum, 1% bovine serum albumin in Dulbecco's modified Eagles medium) was applied for 30 minutes. Primary antibodies were then applied at appropriate dilutions (table 11). Sections were incubated overnight at the appropriate temperature (table 11). Biotinylated secondary antibodies were applied, followed by streptavidin biotin complexes at appropriate dilutions. DAB (Diaminobenzidine) reagent was applied and the sections counterstained with Mayer's haematoxylin and blue.

2.4.3 Neutrophils, Eosinophils and Mast cells

Endogenous peroxidase was inhibited by incubating with 0.1% sodium azide and 0.3% hydrogen peroxide for 30 minutes. Blocking culture medium (20% newborn calf serum, 1% bovine serum albumin in Dulbecco's modified Eagles medium) was applied for 30 minutes. Primary antibodies were then applied as follows: Neutrophil Elastase (NOE) for neutrophil staining (Abcam, Cambridge, UK), AA1 for mast cell staining (Abcam, Cambridge, UK) and EG2 for eosinophil staining (Abcam, Cambridge, UK). Sections were incubated overnight at room temperature. Next a biotinylated rabbit anti-mouse secondary antibody was applied, followed by streptavidin biotin complexes at appropriate dilutions. AEC (3-amino 9-ethylcarbazole) reagent was applied and the sections counterstained with Mayer's haematoxylin and blue.

Table 11: Details of primary and secondary antibodies

Primary antibody and dilution	Secondary antibody	Overnight incubation
Collagen I 1:5000 (Abcam, Cambridge, UK)	Rabbit anti-mouse	Room temperature
Collagen III 1:2000 (Abcam, Cambridge)	Rabbit anti-mouse	Room temperature
α-SMA 1:100,000 (Abcam, Cambridge, UK)	Rabbit anti-mouse	Room temperature
Periostin 1:5000 (Abcam, Cambridge, UK).	Swine anti-rabbit	4°C
EGFR 1:400 (Southampton University, EGFR floss)	Rabbit anti-goat	4°C
$p21^{waf}$ 1:100 (Santa Cruz Biotechnology)	Rabbit anti-mouse	Room temperature
TGF-β2 1:100 (Abcam, Cambridge, UK)	Rabbit anti-mouse	Room temperature
IL-8 1:300 (BioScience)	Rabbit anti-mouse	Room temperature
IL-33 1:150 (Abcam, Cambridge, UK)	Rabbit anti-mouse	Room temperature

2.5 QUANTITATION OF IMMUNOHISTOCHEMICAL STAINING

Each immunostained slide was assessed under a Zeiss light microscope at x10 magnification then compared with the corresponding negative control to ensure correct staining. Both samples were analysed and a mean reading taken.

2.5.1 Collagens I and III and collagen band thickness

The extent of interstitial staining was measured using a computer image analysis program.

The percentage of interstitial collagen I and III immunostaining was assessed using KS400 (Image Associates) software applied to high resolution digital camera captured images of the IHC stained biopsies and expressed as area ratio, based on the red/green/blue colour composition of the DAB staining within the lamina propia (Puddicombe, Polosa et al. 2000). Areas of smooth muscle, glands, epithelium, reticular basement membrane and damaged tissue were excluded.

Collagen band thickness (i.e. thickness of the lamina reticularis) was measured using a computer analysis programme where intact longitudinally orientated epithelium was present using the collagen III sample; if neither of the collagen III samples were satisfactory the Collagen I sample was used (Britten, Howarth et al. 1993).

2.5.2 α-SMA volume

A stereological approach was used to measure the smooth muscle volume fraction. Images were captured at x10 magnification. A point counting grid was superimposed on each image and points overlying submucosal tissue were counted; then points overlying smooth muscle within the submucosal tissue were counted. The volume fraction of smooth muscle was calculated by expressing the number of points falling in smooth muscle points as a fraction of total points falling within the submucosal tissue.

2.5.3 Periostin in Subepithelium

Periostin was analysed as per collagens I and III but due to the localisation of periostin in the subepithelial layer a specific programme was designed by Dr Susan Wilson whereby the analysis was limited to 50µm below the epithelial basement membrane.

2.5.4 EGFR, $p21^{waf}$, Il-8 and in TGF-β_2 in Epithelium

EGFR, $p21^{waf}$, Il-8 and TGF-β_2 staining were analysed using the same method as per collagens I and III but within well-orientated epithelium rather than the subepithelial area.

2.5.5 IL-33 in Epithelium

IL-33 was analysed using the same method as per collagens I and III but the positive staining was expressed using a positive nuclei percentage programme.

2.5.6 Neutrophils, Eosinophils and Mast Cells

Neutrophils, eosinophils and mast cells were analysed using the same method as per collagens I and III. The number of cells positively staining in each biopsy was counted and the number of cells per mm^2 calculated.

2.5.7 Missing data

As far as possible, where staining was inadequate, the biopsy was sampled again and staining repeated until an adequate sample was obtained. In some cases however the amount of biopsy was not sufficient to perform further staining and in these cases data is missing. For this reason sample numbers may differ.

CHAPTER 3 CLINICAL CHARACTERISATION

3.1 DEFINITIONS

A participant was characterised as being asthmatic if they had a history of physician diagnosed asthma plus:

i) Symptoms of wheeze in the last 12 months **and** currently taking regular preventative asthma medication e.g. inhaled corticosteroids/long-acting beta-2 agonist

or

ii) Symptoms of wheeze in the last 12 months **and** using short-acting beta-2 agonist only **and** positive bronchial provocation test

A participant was considered to be in remission of asthma if they had not used regular asthma medication e.g. inhaled corticosteroids/long-acting beta-2 agonist or reliever medication within the last 12 months **and** had a negative mannitol challenge test

A participant was considered atopic if they had one or more positive skin prick tests.

Four groups were defined for the purpose of this study:

1) *Never asthma (NA)*: no asthma at any age

2) *Persistent asthma (PA)*: asthma recorded at or before the 10-year follow up or reported by the participant to have commenced before the age of 10 years and asthma at current visit as per above definition

3) *Asthma remission (AR)*: asthma recorded at or before the 10-year follow up or reported by the participant to have commenced before the age of 10 years and remission of asthma at current visit as per above definition

4) *Intermittent wheezer (IW)*: any participants who did not meet the criteria for the first three categories

3.2 PARTICIPANTS SCREENED

Of the 1456 cohort members 325 sets of notes were screened and 264 invitation letters sent out. In total 61 participants were screened at the David Hide Asthma and Allergy Centre (Isle of Wight) undertaking skin prick testing, questionnaires and bronchial provocation tests with Mannitol. Six participants did not satisfy the inclusion criteria and therefore failed the screening procedure. Fifty five participants were enrolled into the study, 16 with persistent asthma (PA), 19 with asthma remission (AR) and 20 with never asthma (NA). This formed the clinical group who were fully characterised.

11 participants dropped out of the study after visit one, 2 with persistent asthma (12.5% of the group), 5 with asthma remission (26.3% of the group) and 4 in the never asthma group (20% of the group). Those who did not continue with the study were either unable to attend for bronchoscopy due to other commitments (such as work or studying) or did not wish to undertake the procedure.

44 participants attended for bronchoscopy, of those 4 were either unable to tolerate the bronchoscopy or had samples that were insufficient to analyse. Therefore a maximum of 40 bronchial samples were analysed in each section: 12with PA, 14 with AR and 14 with NA (see consort diagram – fissure 6).

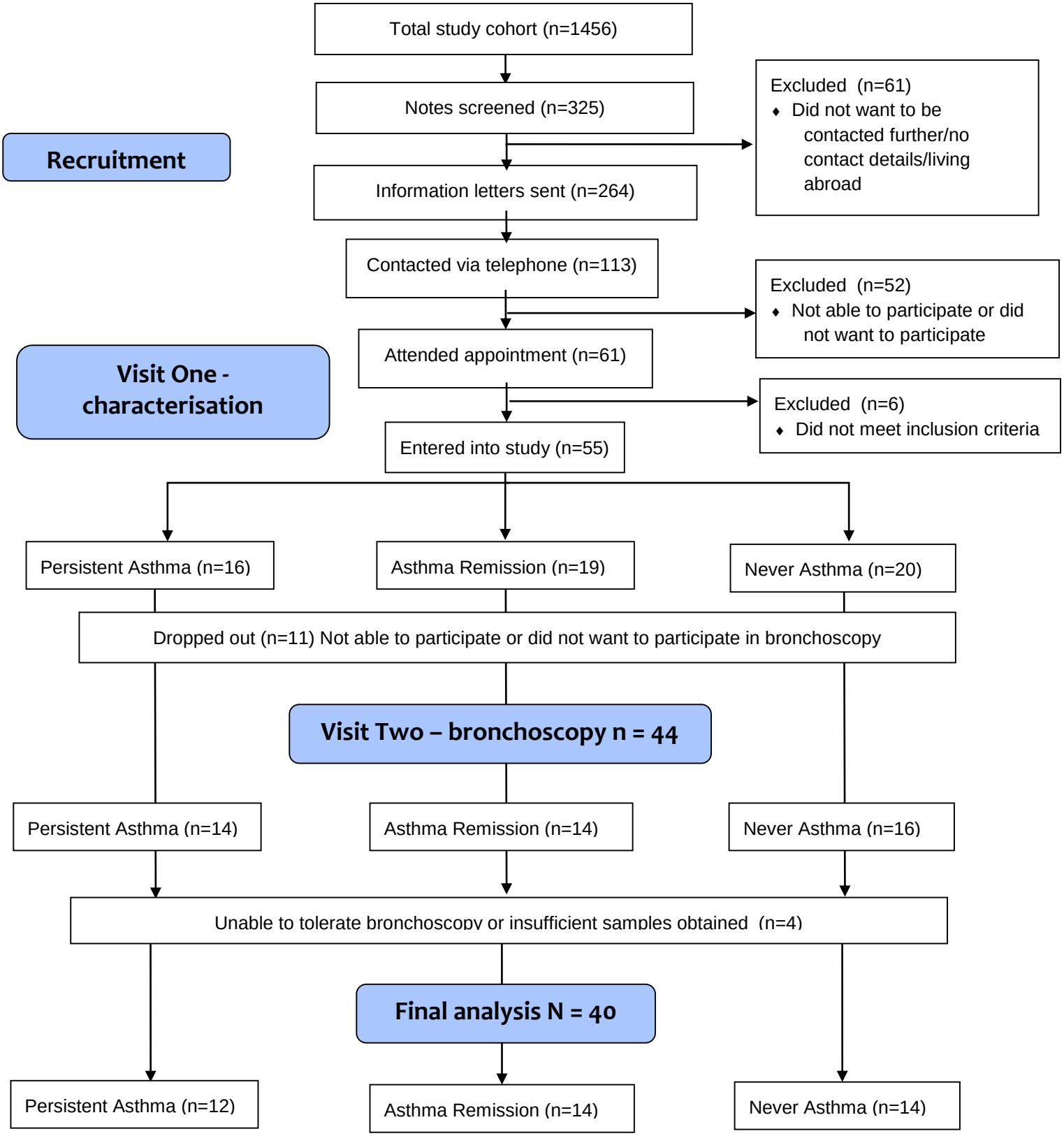


Figure 6: consort diagram to demonstrate recruitment into the study

3.3 GENDER AND ANTHROPOMETRIC MEASUREMENTS

More females than males entered the study in each group (36 female vs 19 male, 65.5% vs 34.5%) (table 5). 56.3% (n = 9) of those with PA were female, 65% (n = 13) of those with AR and 68.4% (n = 14) of those in the NA group.

At the time of giving consent to participate in the study the participants ranged in age from 21 years 8 months to 23 years 9 months. Mean age was 22 years and 6 months.

3.3.1 Height

The mean height for females in the PA group was 163.9cm with a range of 153.8cm to 178.9m (SD: 0.076), for girls in the AR group the mean height was 166.4 cm with a range of 155.3cm to 177.0cm (SD: 0.100) and for females with NA the mean height was 169.6cm with a range of 155.9cm to 189.6cm (SD: 0.095). ANOVA testing revealed that there were no significant differences in height for females between groups (p = 0.599).

The mean height for boys in the PA group was 177.8cm with a range of 168.4cm to 190.8cm (SD: 0.076), for males in the AR group the mean height was 177.9cm with a range of 168.0cm to 191.5cm (SD: 0.100) and for males with NA the mean height was 181.6cm with a range of 174.2cm to 189.6cm (SD: 0.055). ANOVA testing revealed that there were no significant differences in height for males between groups (p = 0.634).

3.3.2 Weight

The mean weight for females in the PA group was 71.2kg with a range of 51.1kg to 103.2kg (SD: 14.57); for females in the AR group the mean weight was 74.1kg with a range of 48.5kg to 110.2kg (SD: 22.50) and for females with NA the mean weight was 71.2kg with a range of 48.5kg to 110.0kg (SD: 17.12). ANOVA testing revealed that there were no significant differences in weight for females between groups (p = 0.728) (figure 7a).

The mean weight for males in the PA group was 78.4kg with a range of 64.6kg to 98.8kg (SD: 12.119); for boys in the AR group the mean weight was 85.0kg with a range of 65.7kg to 116.0kg (SD: 18.839) and for males with NA the mean weight was 83.4kg with a range of

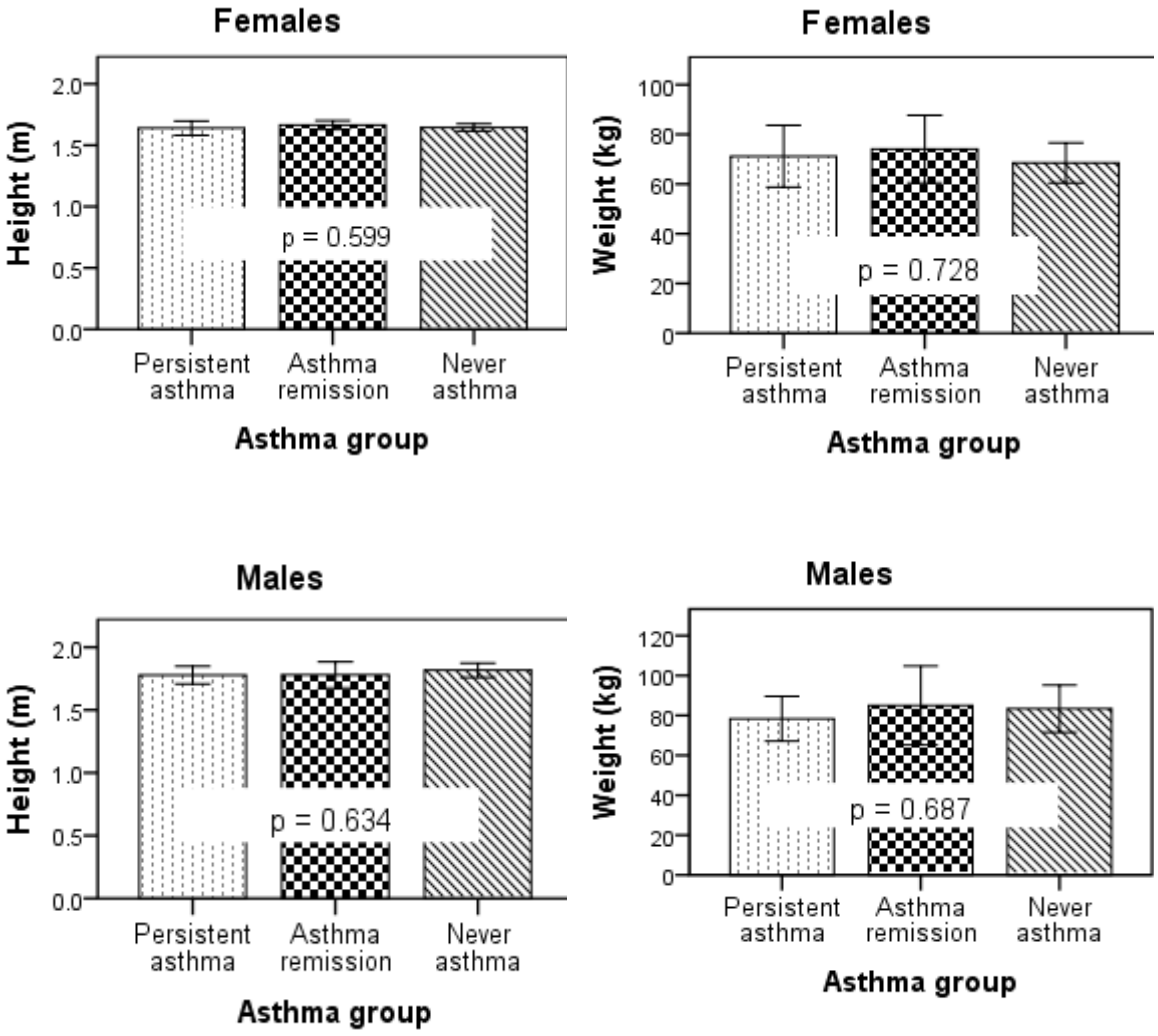
66.0kg to 96.1kg (SD: 11.258). ANOVA testing revealed that there were no significant differences in weight for males between groups (p = 0.687) (figure 7a).

3.3.3 Body Mass Index

Analysis of BMI revealed a significant outlier (female with AR) with a BMI of 45 (see figure 7b). This patient was excluded from the analysis and the results are presented as for normally distributed date. Mean BMI for females overall was 25.71kg/m^2 (range 18.95 to 39.3; SD: 6.32) and for boys overall was 25.61kg/m^2 (range 18.62 to 34.07; SD: 4.11). Within each asthma group the mean BMI for those with PA was 25.94kg/m^2, (range 18.62 to 37.72; SD: 5.89), 26.23kg/m^2 (range 18.95 to 39.30; SD: 6.44) for those with AR and 25.37 kg/m^2 (range 19.34 to 36.12; SD: 4.91) for those in the NA group. ANOVA testing revealed that there were no significant differences in BMI between groups (p = 0.872).

Figure 7a: Mean height (m) and weight (kg) stratified by sex in each asthma group.

Error bars represent 95% confidence intervals.



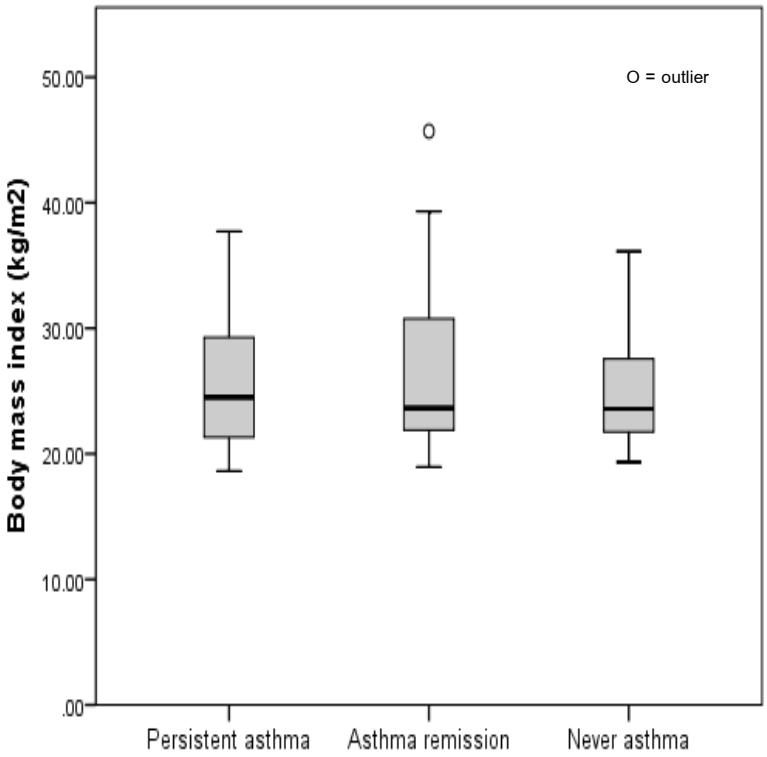


Figure 7b. Body mass index (kg/m^2) by asthma group

3.4 SOCIOECONOMIC INDICES

3.4.1 Employment and Educational Status

In total 43 out of 55 participants were in full time employment (78.2%), 4 were unemployed (7.3%), 5 in full time education (9.1%) and 3 in education and part-time employed (5.5%).

Analysis using Fisher's exact test revealed no significant differences between asthma groups ($p = 0.123$), the percentages by asthma group are summarised in table 5.

3.4.2 Tenure of Housing

In total 31 out of 55 participants were living in privately owned accommodation (56.4%), 17 were living in privately rented accommodation (30.9%), 5 were living in in council housing (9.1%) and 2 (3.6%) classified their living arrangements as 'other'.

Analysis using Fisher's exact test revealed no significant differences between asthma groups ($p = 0.837$), the percentages by asthma group are summarised in table 5.

3.4.3 Family Income

Total family income was divided into bands of <£12,000, £12 – £17,999, £18 - £29,999, £30 – £42,000 and >£42,000 in line with previous questionnaires used. 8 out of 55 participants reported their 'family' income as being <£12,000 (14.5%), 9 as £12 – £17,999 (20%), 12 as £18 - £29,999 (18.5%), 6 as £30 – £42,000 (10.9%) and 10 as >£42,000 (16.4%).

Analysis using Fisher's exact test revealed no significant differences between asthma groups ($p = 0.148$), the percentages by asthma group are summarised in table 5. It is worth noting that a significant proportion of participants were unable to answer this question (20%).

Table 5: Characteristics of participants entering the study

	Asthma group						Total (and % per asthma group)	
	Persistent asthma n = 16		**Asthma remission n = 19**		**Never asthma n = 20**			
Sex								
Male	7	43.8%	6	35%	6	31.6%	19	34.5%
Female	9	56.3%	13	65	14	68.4%	36	65.5%
Employment status								
Employed	14	87.5%	13	68.4%	16	80%	43	78.2%
Unemployed	0	0%	4	21.1%	0	0%	4	7.3%
In education	2	12.5%	1	5.3%	2	10%	5	9.1%
In education and part- time employment	0	0%	1	5.3%	2	10%	3	5.5%
Smoking status								
Current smoker	4	25%	9	47.4%	4	20%	17	30.9%
Ex-smoker	1	6.3%	0	0%	0	0%	1	1.8%
Never smoker	11	68.8%	10	47.4%	15	75%	35	63.6%
Occasional smoker	0	0%	1	5.3%	1	5%	2	3.6%
Family income								
<£12,000	3	18.8%	3	15.8%	2	10%	8	14.5%
£12-£17,999	7	43.8%	1	5.3%	3	15%	9	20%
£18-£29,999	0	0%	5	26.3%	5	25%	12	18.5%
£30-£42,000	2	12.5%	2	10.5%	2	10%	6	10.9%
>£42,000	1	6.3%	3	15.8%	5	25%	10	16.4%
Don’t know	3	18.8%	5	26.3%	3	15%	11	20%
Accommodation								
Privately owned	11	68.8%	9	47.4%	11	55%	31	56.4%
Privately rented	4	25%	6	31.6%	7	35%	17	30.9%
Council house	1	6.3%	3	15.8%	1	5%	5	9.1%
Other	0	0%	1	5.3%	1	5%	2	3.6%

3.4.4 Smoking Status

In total 17 of 55 participants (30.9%) of the group were current smokers, 1 was an ex-smoker (1.8%), 35 were never smokers (63.3%) and 2 were occasional smokers (3.6%). Analysis using Fisher's exact test revealed no significant differences between asthma groups ($p = 0.233$), the percentages by asthma group are summarised in table 5.

Exposure to smoking in the home was analysed only for those participants who did not smoke themselves (i.e. never or ex-smoker). Of 37 never or ex-smokers 5 participants (13.5%) were exposed to passive smoke within their home.

3.4.5 Leisure Activities

Throughout all groups the average number of hours for which a participant watched television per week was 13.6 hours (range 10 - 50) and the average number of hours using a computer was 19.4 hours (range 0 - 84). One way ANOVA analysis revealed no significant differences between groups for either hours watching television ($p = 0.193$) or hours using a computer ($p = 0.260$).

3.5 EXPOSURE TO ALLERGENS AND POLLUTION

3.5.1 Air and Traffic Pollution

In total 11 of 55 participants (20%) were exposed to high levels of air pollution in their home, the highest percentage were in the AR and NA groups (>80%) whereas the PA group had a lower exposure at 68.8%; however analysis using Fisher's exact test revealed no significant differences between asthma groups (p = 0.310)(table 6). Traffic exposure appeared to differ between groups with 75% of those with PA being exposed to low levels whereas in the other two groups the proportion was reversed with 63.2% of the AR group and 55% of the NA group being exposed to high levels of traffic pollution. Despite this the p value 0.064 on Chi square analysis did not suggest a significant difference between groups.

3.5.2 Pets

Most (92.7%) participants were exposed to some sort of pet in the home environment on a regular basis. The most common pets were cats (54.5%), followed by dogs (18.2%), while 12.7% were exposed to multiple pets at home. 7.3% had *other* pets such as hamsters, gerbils and snakes. Analysis using Fisher's exact test revealed no significant differences between asthma groups (p = 0.863), the percentages by asthma groups are summarised in table 6.

3.5.3 Moulds and Fungus

Of 55 participants, 16 (29.1%) reported being potentially exposed to mould or fungal spores in their home, this was equally distributed between asthma groups. Analysis using Fisher's exact test revealed no significant differences between asthma groups (p = 0.944), the percentages by asthma groups are summarised in table 6.

Table 6: Exposure to allergens and pollutants

	Asthma group						Total (and % per asthma group)	
	Persistent asthma n = 16		**Asthma remission n = 19**		**Never asthma n = 20**			
Air pollution								
Low	11	68.8%	17	89.5%	16	80%	44	80%
High	5	31.3%	2	10.5%	4	20%	11	20%
Traffic pollution								
Low	12	75%	7	36.8%	9	45%	28	50.9%
High	4	25%	12	63.2%	11	55%	27	49.1%
Pets in the home								
None	1	6.3%	2	10.5%	1	5%	4	7.3%
Cat	11	68.8%	9	47.4%	10	50%	30	54.5%
Dog	3	18.8%	4	21.1%	3	15%	10	18.2%
Multiple	1	6.3%	2	10.5%	4	20%	7	12.7%
Other	0	0%	2	10.5%	2	10%	4	7.3%
Mould or fungus								
Yes	5	31.3%	5	26.3%	6	30%	16	29.1%
No	11	68.8%	14	73.7%	14	70%	39	70.95

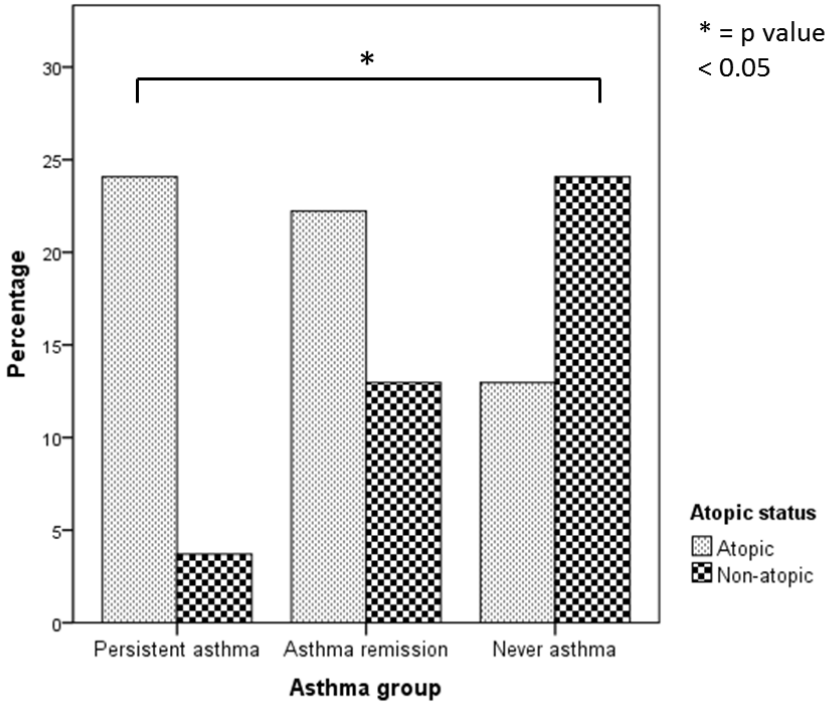
3.6 ATOPY

3.6.1 Atopic Status and Skin Prick Testing

Atopipc status was defined by one or more positive skin prick tests. Overall 59.3% (n = 32) of the particpants were atopic. In the group with PA 86.7% (n = 13) were atopic, in the group with AR 63.2% (n = 12) were atopic and in the NA group 35% (n = 7) were atopic (see figure 8). In total 12 males were atopic (63.2%) and 20 females (55.6%).

Analysis using Fisher's exact test showed that those with persistent asthma were significantly more likely to be atopic than those with NA (p = 0.005)(figure x). Those with AR were intermediate between the two, having lower levels of atopy than those with PA and higher than those with NA but with no significant difference to either those with PA (p=0.240) or those with NA (p=0.113) (figure 8).

Figure 8. Atopic status stratified by asthma group



3.6.2 Co-morbidity with other Allergic Disease

More participants from the PA and AR groups (31.3% and 26.3%) reported current eczema than in the NA group (15%); however the numbers were small and no significant difference (p=0.527 by Fisher's exact test) was demonstrated between the groups.

More participants from the PA group (43.8%) reported current food allergy than from the AR and NA group (15.8% and 10%). Again the numbers were quite small but the p value looking for an overall difference of 0.052 (by Fisher's exact test) was approaching significance.

A large percentage of those with PA (93.8%) reported current hayfever, 52.6% of those with AR did so and 30% of those with NA. Fisher's exact test demonstrated that the difference between the PA and NA groups was significant ($p<0.001$) as was the difference between PA and AR groups (p=0.012). The difference between the AR group and NA group was not significant (p=0.299).

Of the 34 participants who described *current hayfever*; 15 (44.1%) reported that hayfever symptoms bothered them 'not at all,' 12 (35.3%) reported 'a little,' 7 (20.6%) reported 'a moderate amount' and no-one reported that their symptoms bothered them 'a lot.' Of the 12 participants who reported *current eczema*; 9 (75%) reported that eczema symptoms 'never' kept them awake, 1 (8.3%) reported 'less than one night a week' and 2 (16.7%) reported 'more than one night per week.' Only 1 participant described suffering with urticaria (1.8%), who was from the PA group.

3.7 FEV_1 AND AIRWAY OBSTRUCTION

3.7.1 Percentage predicted FEV_1

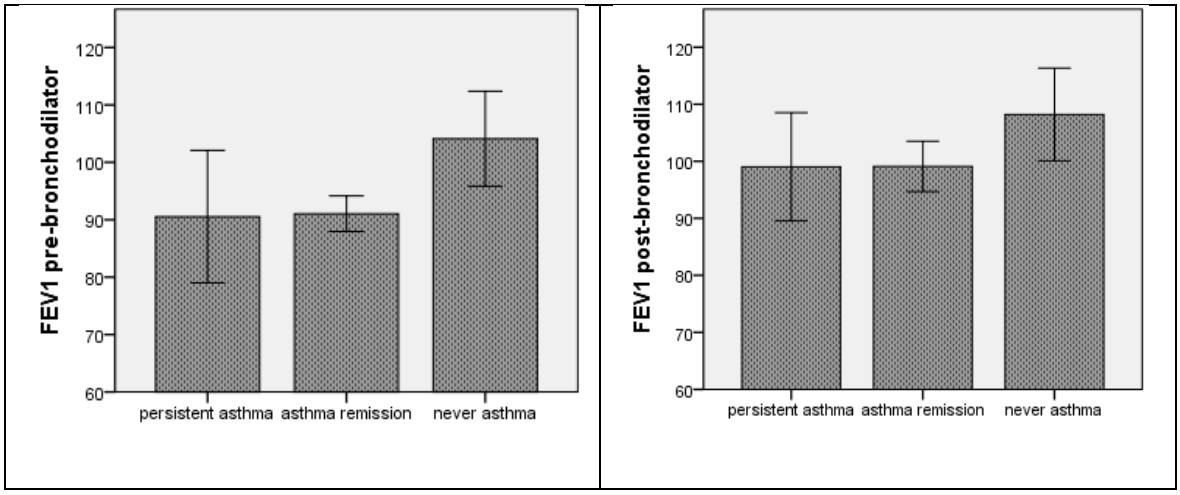
All participants (55) undertook spirometry at Visit two prior to bronchoscopy. It is worth restating that the participants with PA were assessed whilst taking their asthma medication. They were given 2.5mg of Salbutamol via a nebuliser and FEV_1 reperformed.

Mean pre-bronchodilator percentage predicted FEV_1 for those with PA was 90.5% (range 61.2 to 121.0; SD: 18.2), for AR it was 91.1% (range 82.4 to 99.4: SD: 5.4) and for NA 104.1% (range 83.3 to 127.6; SD: 14.8)(figure 9).

ANOVA revealed a difference between groups with significance of p=0.018. Post-hoc analysis showed that the FEV_1 of those with PA was significantly lower than those with NA (p=0.043; 95% CI: -25.39 to -8.40). The FEV_1 of those with AR was also significantly lower than those with NA (p = 0.005; 95% CI: -21.6 to -4.4).

Mean post-bronchodilator percentage predicted FEV_1 for those with PA was 90.0% (range 75.4 to 122.8; SD: 14.9), for AR it was 99.1% (range 84.5 to 112.4: SD: 7.6) and for NA 108.2% (range 85.3 to 130.5; SD: 14.7)(figure 9). ANOVA revealed no significant difference between groups post-bronchodilator.

Figure 9. Percentage FEV_1 compared between asthma groups pre- and post-bronchodilator with significance values. Bars indicate 95% Confidence intervals.



This study did not include small airways function as part of the analysis. As asthma affects the small airways before the large airways (i.e. a decrease in the FEF_{25-75} or 'scalloping' of the flow-volume loop) looking at the function of the small airways may have been more sensitive in detecting differences between the groups. Such analysis will be considered for analysis in a post-thesis project.

3.7.2 Airway obstruction

Airway obstruction is defined as FEV_1/FVC ratio of <70%. Mean FEV_1/FVC ratio in those with PA was 79.0%, in those with RA was 84.4% and in those with NA was 86.8%. Only 2 participants had a ratio of <70% and both were from the PA group.

3.8 USE OF PARACETAMOL AND IBUPROFEN

The mean number of times that a participant used paracetamol and ibuprofen per month is summarised in table 7. One way ANOVA testing revealed no significant difference between groups with regards to paracetamol and ibuprofen use.

Table 7: Use of Paracetamol and Ibuprofen by asthma group

	Mean (times used/month)	Standard deviation	Minimum	Maximum
Paracetamol				
Persistent asthma	2.4	4.79	0	20
Asthma remission	2.4	4.00	0	15
Never asthma	1.5	3.45	0	15
One way ANOVA p = 0.733				
Ibuprofen				
Persistent asthma	2.9	5.27	0	20
Asthma remission	0.95	1.96	0	8
Never asthma	1.25	3.35	0	15
One way ANOVA p = 0.266				

3.9 CHARACTERISATION OF ASTHMATIC PARTICIPANTS

3.9.1 Use of Asthma Medication and Asthma Severity

16 participants with persistent asthma were recruited to the study. The mean age of developing asthma was 3 years and 3 months (range 0-8 years). Participants were classified according to BTS guidelines as to the severity of their asthma (see methods)(table 8). 5 were at stage 1 (31.3%), 5 at stage 2 (31.3%) and 6 at stage 3 (37.5%). There were no participants recruited from BTS stages 4 and 5.

All participants were using asthma medication (table x); 5 (31.3%) were using an inhaled SABA only, 5 (31.3%) were using a SABA and ICS, 5 (31.3%) were using a SABA, ICS and an inhaled LABA and 1 (6.3%) was using a LABA and ICS without SABA (this is not recommended by the BTS).

3.9.2 Asthma Symptoms and Impact on Quality of Life

All participants answered questions about triggers for their asthma. The results are summarised in table 8. The most common trigger was pollen (n = 14, 87.5%) and the least common stress (n = 5, 37.5%). All participants reported two or more triggers for their asthma with 2 (12.5%) reporting two triggers, 5 (31.3%) reporting three triggers, 3 (18.8%) reporting four triggers, 4 (25%) reporting five triggers and 2 (12.5%) reporting six triggers.

All participants reported the number of wheezing episodes in the previous year and all 16 had at least one episode of wheezing. 7 (43.8%) reported between one and three episodes of wheezing, 4 (25%) reported 4-12 episodes of wheezing and 5 (31.3%) reported more than 12 episodes of wheezing. Only 3 (18.8%) reported never waking at night (in the previous year) with wheezing, 6 (37.5%) reported waking less than one night per week with wheezing and 7 (43.8%) reported waking more than one night per week with wheezing (table 8). 6 of 16 participants (37.5%) reported at least one episode of asthma severe enough to limit their speech within the previous year.

Only 3 participants (18.8%) had required time away from work or education due to their asthma symptoms in the previous year. The remaining 12 (81.2%) had missed no work or education. The mean number of asthma attendances to hospital in the previous 12 months

was 0.6 (range 0-4) with 12 out of 16 participants (81.2%) not needing to attend hospital with asthma symptoms in the previous 12 months.

3.10 CHARACTERISATION OF REMISSION PARTICIPANTS

Mean age of onset of asthma in the AR group was 3.2 years (median of 2 years, range 0 - 9 years). Mean age of growing out of asthma was 16.3 years (median of 17 years). This means that mean length of having asthma was 13.1 years (median of 14 years).

Table 8: Characteristics of asthma participants		
	Number	
BTS stage		
Stage 1: intermittent asthma	5	31.3%
Stage 2: regular preventer therapy:	5	31.3%
Stage 3: initial add-on therapy	6	37.5%
Stage 4: persistent poor control	0	0%
Stage 5: regular oral steroids	0	05
Use of asthma medications		
SABA	5	31.3%
SABA + ICS	5	31.3%
SABA + LABA + ICS	5	31.3%
LABA + ICS	1	6.3%
Triggers of asthma symptoms		
Pollen	14	87.5%
Stress	6	37.5%
Exercise	11	68.8%
Infection	11	68.8%
House dust	12	75%
Animals	8	50%
Number of triggers reported		
1	0	0%
2	2	12.5%
3	5	31.3%
4	3	18.8%
5	4	25%
6	2	12.5%
Wheezing episodes in last 12 months		
None	0	0%
1-3	7	43.8%
4-12	4	25%
>12	5	31.3%
Sleep disturbance in last 12 months		
Never woken with wheezing	3	18.8%
< 1 night/wk	6	37.5%
> 1 night/wk	7	43.8%

CHAPTER 4 BRONCHIAL HYPER-RESPONSIVENESS, AIRFLOW REVERSIBILITY AND MARKERS OF INFLAMMATION

4.1 ASSESSMENT OF BRONCHIAL HYPER-RESPONSIVENESS (MANNITOL CHALLENGE)

In total 53 participants were successfully screened and underwent assessment of bronchial hyper-responsiveness BHR using a Mannitol challenge as described in the methods chapter; 14 with PA, 19 with AR and 20 with NA. 2 participants with PA were unable to prepare adequately for the test as their asthma symptoms would not allow them to stop their inhaler therapy as required.

All participants with AR and NA had a negative Mannitol test i.e. no evidence of BHR (as part of inclusion criteria). 2 participants with PA had a negative Mannitol test and the remaining 11 had a positive test (table 9).

Table 9. Result of mannitol challenge test by asthma group

	BHR status					
	Positive		**Negative**		**Unable to perform**	
Asthma group	N		N		N	
Persistent	12	75%	2	12.5%	2	12.5%
Remission	0	0%	19	100%	0	0%
Never	0	0%	20	100%	0	0%

The participants from the NA and AR groups (all of whom had a negative Mannitol challenge test) had their FEV_1 before and after the procedure compared to see what percentage drop in FEV_1 occurred. Mean drop in those with AR was 3.68% and in those with NA was 0.43%. This we analysed using non-parametric testing (Mann Whitney U) and found to be significantly different ($p = 0.007$), therefore although no participants reached the significant cut off of 15% drop in FEV_1 those with AR had a higher drop in FEV_1 than those with NA.

Had the participants in this study been tested using Methacholine (a direct bronchoconstrictor), this may have led to different findings. Methacholine is reported to have a higher sensitivity than indirect agents (O'Byrne and Zamel 1995, Cockcroft 2001) and therefore a negative test is used to exclude asthma, whereas Mannitol is reported as being more specific (Brannan, Anderson et al. 2005). Testing with Methacholine may have led to a higher detection of BHR in the NA and AR groups but this may not have been representative of underlying disease. For this reason we chose to use Mannitol due to its direct effects on stimulating the airways which is more representative of the inflammatory process that occurs in active asthma.

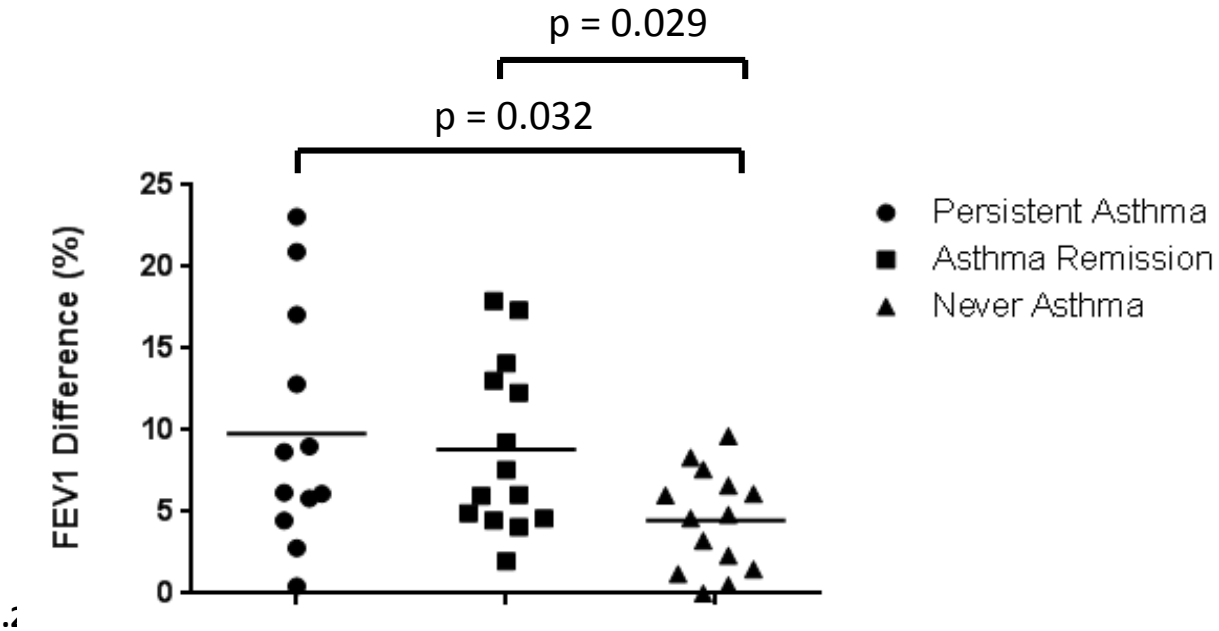
4.2 AIRFLOW REVERSIBILITY

Airflow reversibility to Salbutamol was tested in 41 participants, 12 with PA, 14 with AR and 15 with NA (Table 10). Mean reversibility (expressed as % difference in FEV_1 between first FEV_1 reading and FEV_1 after administration of Salbutamol) was analysed between groups using non-parametric analysis (Kruskal Wallis) which revealed a p value of 0.039 indicating that there were significant differences between the groups. Those with PA and AR had significantly higher reversibility than those with NA (p = 0.032 and p = 0.029 respectively) (figure 10).

Table 10: Mean FEV_1 reversibility (%) with significance values

Asthma group	Mean FEV_1 difference (%)	Number	SD
Persistent asthma	9.53	12	7.40
Asthma remission	8.83	14	5.20
Never asthma	4.16	15	3.18

Figure 10: Dot plot demonstrating mean FEV_1 reversibility (%) with significance values (Horizontal line represents mean)



4.3 MARKERS OF INFLAMMATION

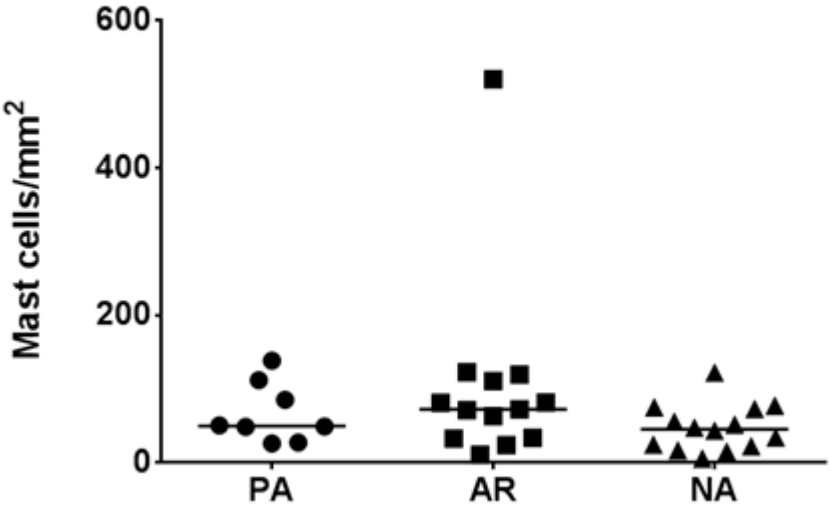
4.3.1 Mast Cells

Mast cells were counted and calculated as cells/mm^2 in 8 subjects with PA, 13 with AR and 14 with NA. Median readings in the PA group were 49.92cells/mm^2 (IQR 72.76), in the AR group 72.46cells/mm^2 (IQR 81.89) and in the NA group 45.42cells/mm^2 (IQR 52.54) (table 12). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated non-significance between the groups (figure 11).

Table 12: Median mast cells/mm^2 with IQR and significance testing

	Median	**IQR**	**n**
Persistent Asthma	49.92	72.76	8
Asthma Remission	72.46	81.89	13
Never Asthma	45.42	52.54	14
Between groups Kruskal Wallis test		p = 0.170	

Figure 11: Dot plot of mast cells/mm^2 (horizontal line represents median)



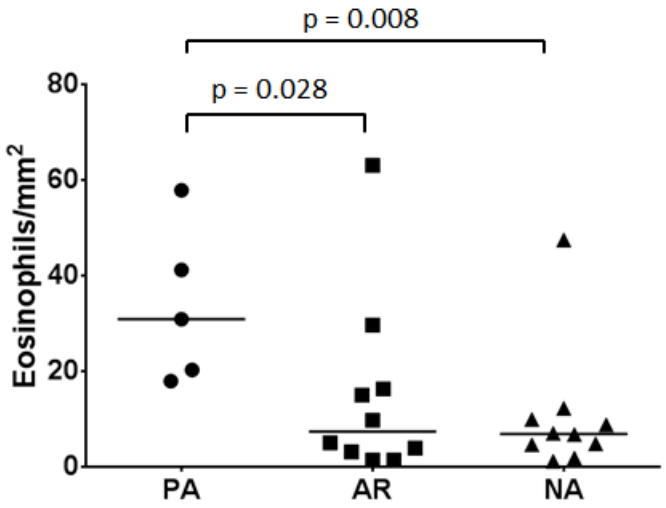
4.3.2 Eosinophils

Eosinophils were counted and calculated as cells/mm^2 in 5 subjects with PA, 10 with AR and 11 with NA. Median readings in the PA group were 30.91cells/mm^2 (IQR 30.43) in the AR group 7.40cells/mm^2 (IQR 16.95) and in the NA group 6.76cells/mm^2 (IQR 8.07) (table 13). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of 0.026 indicating significant differences between the groups (figure 12). Those with PA had significantly more eosinophils than those with AR (p = 0.027) and those with NA (p = 0.008).

Table 13: Median eosinophils/mm^2 with IQR and significance testing

	Median	**IQR**	**n**
Persistent Asthma	30.91	30.43	5
Asthma Remission	7.40	16.95	10
Never Asthma	6.76	8.07	11
Between groups Kruskal Wallis test		p = 0.026	
Asthma Persistence vs Asthma Remission		p = 0.027	
Asthma Persistence vs Never Asthma		p = 0.008	
Never Asthma vs Asthma Remission		p = 0.526	

Figure 12: Dot plot of eosinophils/mm^2 (horizontal line represents median)



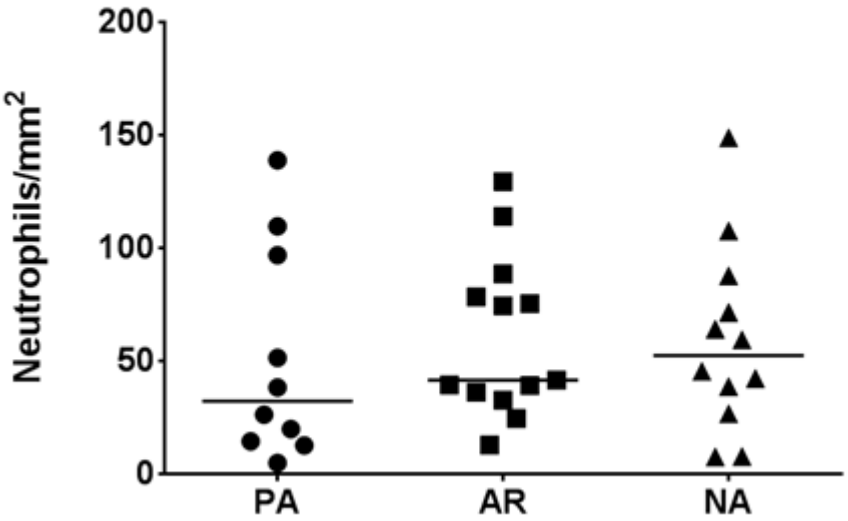
4.3.3 Neutrophils

Neutrophils were counted and calculated as cells/mm^2 in 10 subjects with PA, 13 with AR and 12 with NA. Median readings in the PA group were 51.53cells/mm^2, in the AR group 60.70cells/mm^2 and in the NA group 59.17cells/mm^2 (table 14). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated non-significance between the groups (figure 13).

Table 14: Median neutrophils/mm^2 with IQR and significance testing

	Median	**IQR**	**n**
Persistent Asthma	32.47	86.06	10
Asthma Remission	41.67	49.10	13
Never Asthma	52.61	53.97	12
Between groups Kruskal Wallis test		p = 0.676	

Figure 13: Dot plot of neutrophils/mm^2 (horizontal line represents median)



CHAPTER 5 MARKERS OF REMODELLING

5.1 COLLAGEN BAND THICKNESS

Thickness of the lamina reticularis, referred to here as collagen band thickness (CBT) was measured in 9 subjects with PA, 12 with AR and 9 with NA. Median CBT in the PA group was 13.21µm (IQR 4.83), in the AR group was 11.65µm (IQR 3.47) and in the NA group was 8.48µm (IQR 1.99). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of 0.001 between groups (table 15). Further analysis revealed that those with PA and AR had significantly higher CBT than those with NA ($p<0.001$ and $p=0.001$ respectively). The CBT between those with PA and AR was not significantly different.

5.2 SUBEPITHELIAL COLLAGEN I DEPOSITION

Subepithelial collagen I deposition (%) was measured in 12 subjects with PA, 14 with AR and 13 with NA. Median collagen I deposition in the PA group was 44.73% (IQR 11.04), in the AR group was 34.70% (IQR 18.77) and in the NA group was 32.37% (IQR 11.51). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of 0.009 between groups (table 16). Further analysis revealed that those with PA had a significantly higher collagen I deposition (%) than those with NA ($p=0.001$). Collagen I deposition (%) in those with AR was intermediate between those with PA and NA but did not differ significantly from either.

5.3 SUBEPITHELIAL COLLAGEN III DEPOSITION

Subepithelial collagen III deposition (%) was measured in 12 subjects with PA, 14 with AR and 14 with NA. Median collagen III deposition in the PA group was 46.98% (IQR 8.30), in the AR group was 38.05% (IQR 8.05) and in the NA group was 34.69% (IQR 9.38). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of 0.007 between groups (table 17). Further analysis revealed that those with PA had a significantly higher collagen III deposition (%) than those with AR ($p=0.014$) and those with NA ($p<0.001$). Those with AR also had significantly higher collagen III deposition (%) than those with NA ($p=0.035$).

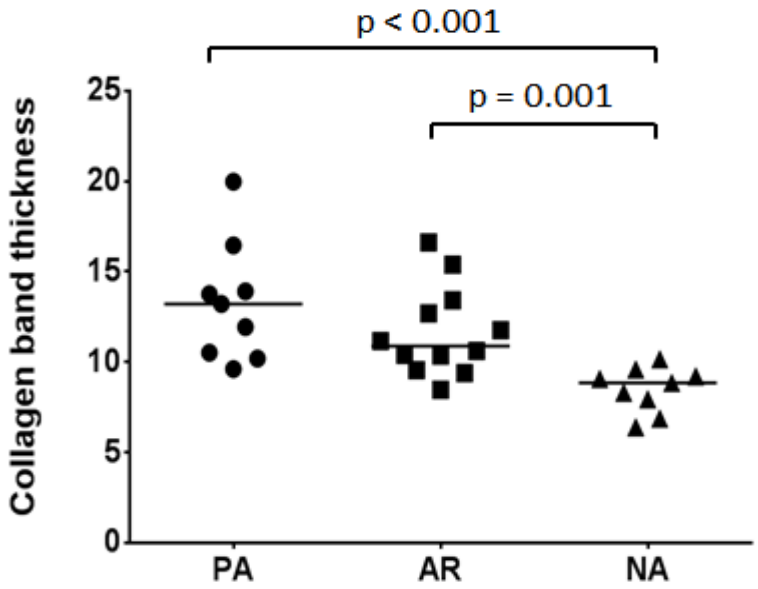
5.4 SUBEPITHELIAL PERIOSTIN

Subepithelial periostin deposition (%) was measured in 8 subjects with PA, 13 with AR and 11 with NA. Median periostin deposition in the PA group was 39.67% (IQR 7.38), in the AR group was 31.15% (IQR 11.53) and in the NA group was 21.65% (IQR 6.84). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of <0.001 between groups (table 18). Further analysis revealed that those with PA had a significantly higher periostin deposition (%) than those with AR (p=0.025) and those with NA (p=0.002). Those with AR also had significantly higher periostin deposition (%) than those with NA (p=0.014).

5.5 ALPHA SMOOTH MUSCLE ACTIN (A SMA)

α-SMA was measured in 11 subjects with PA, 13 with AR and 13 with NA. Median α-SMA deposition in the PA group was 24.30% (IQR 24.42), in the AR group was 20.64% (IQR 10.80) and in the NA group was 17.32% (IQR 14.79). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated non-significance between the groups (table 19).

Table 15: Collagen band thickness

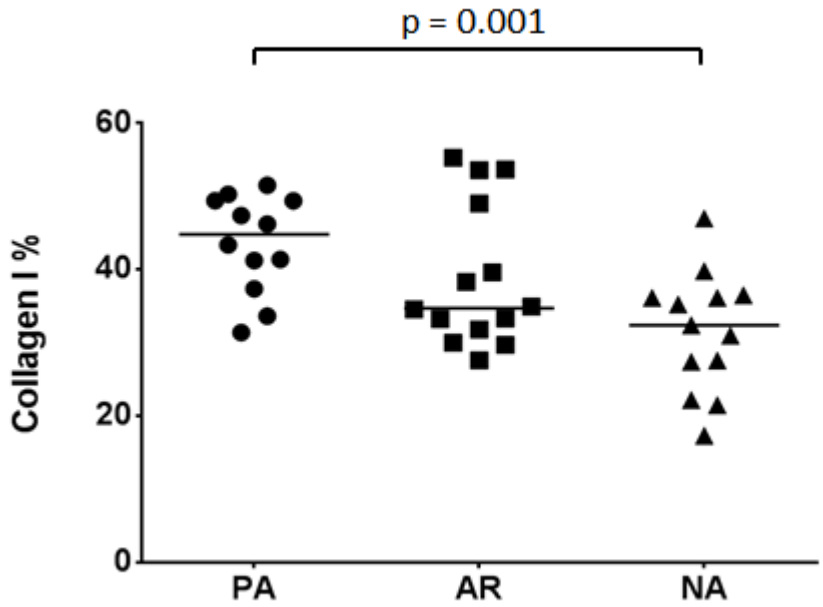


Dot plot of collagen band thickness (µm) by asthma group (horizontal line indicates median)

	Median	**IQR**	**n**
Persistent Asthma	13.21	4.83	9
Asthma Remission	10.89	3.47	12
Never Asthma	8.84	1.99	9

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.001	Reject the Null hypothesis
Asthma Persistence vs Asthma Remission		p = 0.247
Asthma Persistence vs Never Asthma		p <0.001
Never Asthma vs Asthma Remission		p = 0.001

Table 16: Subepithelial collagen I deposition

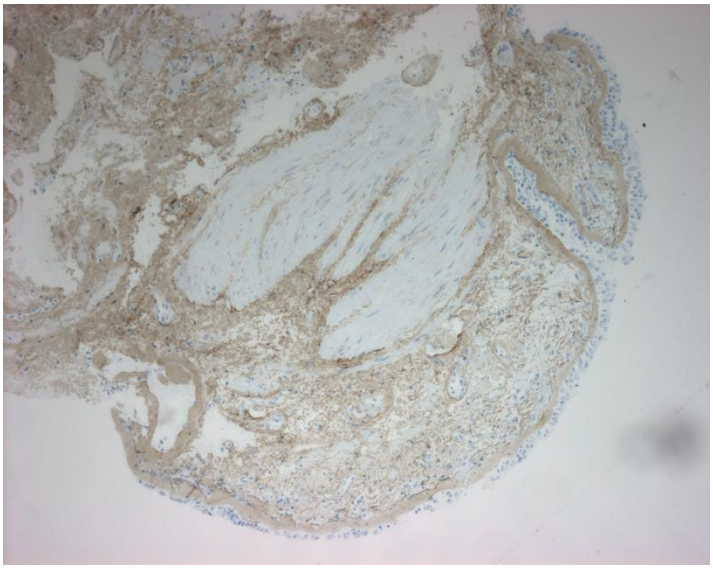


Dot plot of collagen I % by asthma group (horizontal line indicates median)

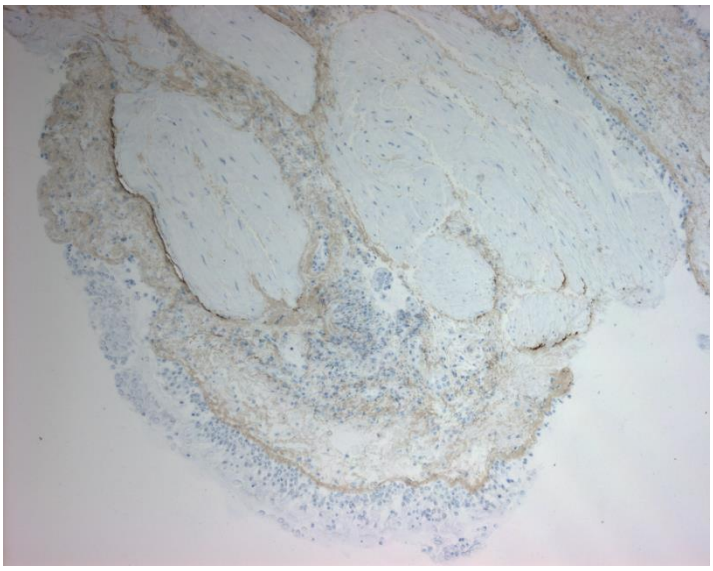
	Median	**IQR**	**n**
Persistent Asthma	44.73	11.04	12
Asthma Remission	34.70	18.77	14
Never Asthma	32.37	11.51	13

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.009	Reject the Null hypothesis

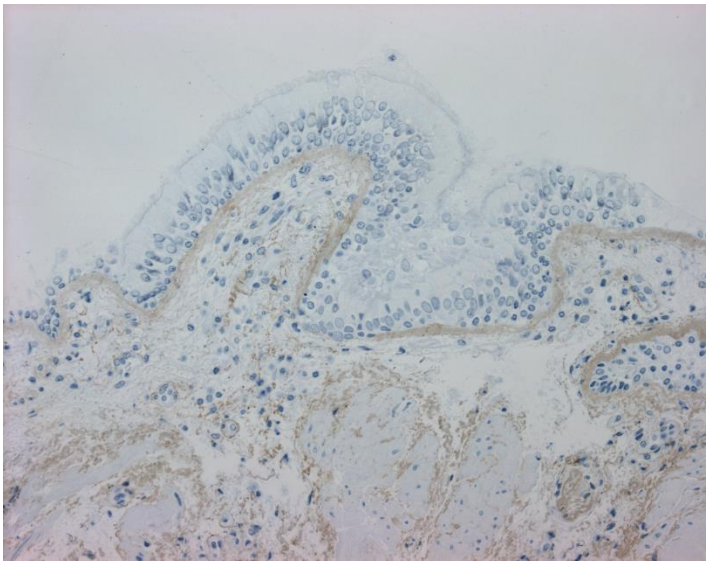
Asthma Persistence vs Asthma Remission	p = 0.165
Asthma Persistence vs Never Asthma	p = 0.001
Never Asthma vs Asthma Remission	p = 0.120



Collagen I in Persistent Asthma

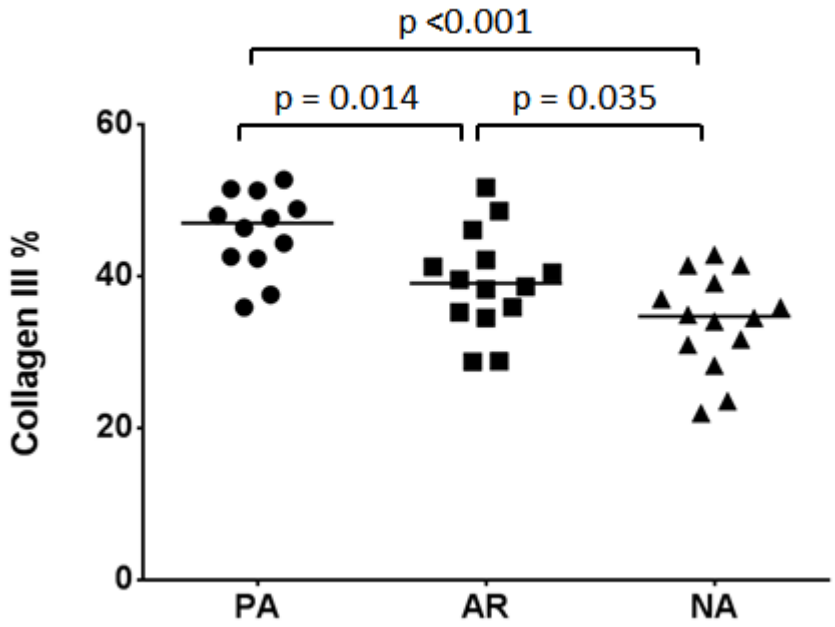


Collagen I in Asthma Remission



Collagen I in Never Asthma

Table 17: Subepithelial collagen III deposition

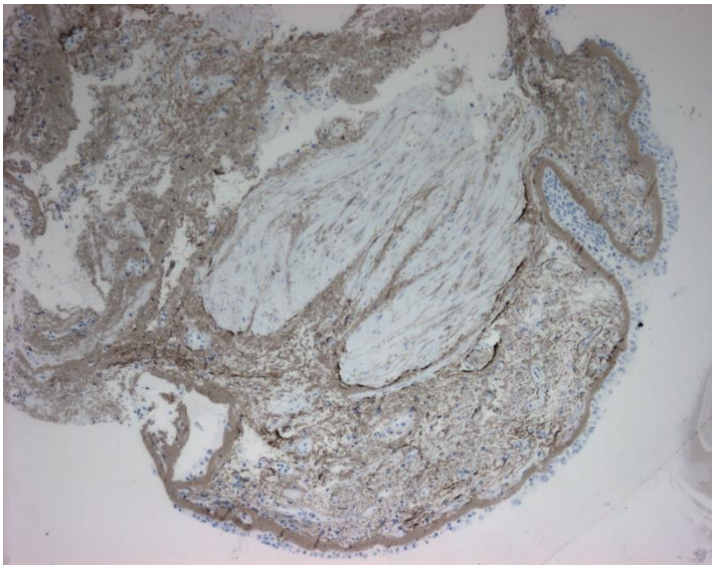


Dot plot of collagen III % by asthma group (horizontal line indicates median)

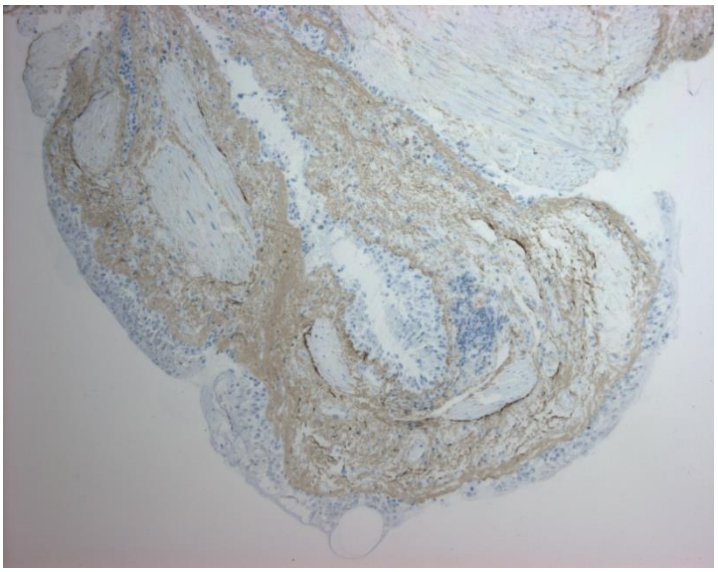
	Median	**IQR**	**n**
Persistent Asthma	46.98	8.30	12
Asthma Remission	39.05	8.05	14
Never Asthma	34.69	9.38	14

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.007	Reject the Null hypothesis

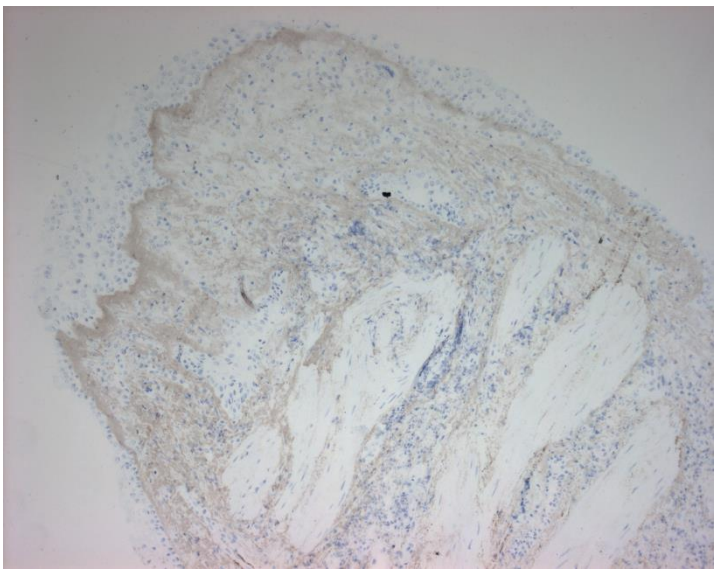
Asthma Persistence vs Asthma Remission	p = 0.014
Asthma Persistence vs Never Asthma	p = <0.001
Never Asthma vs Asthma Remission	p = 0.035



Collagen III in Persistent Asthma

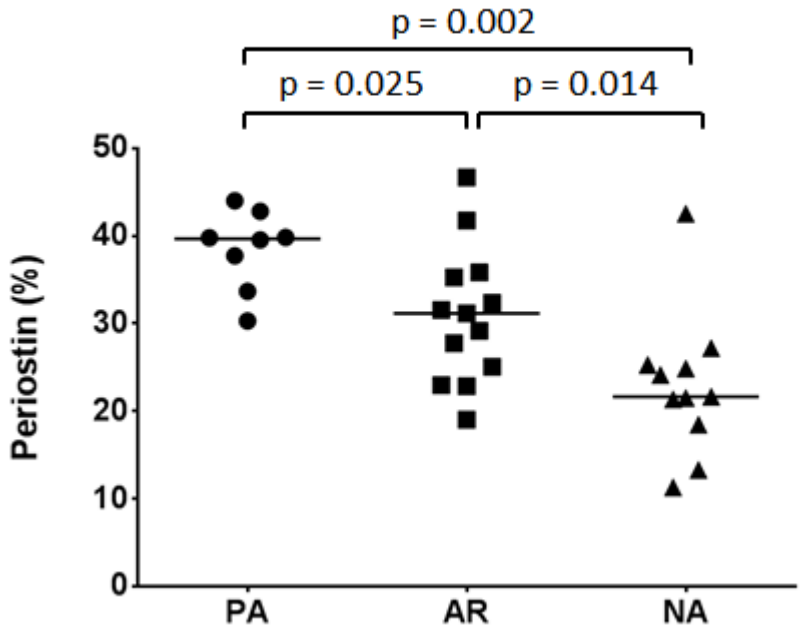


Collagen III in Asthma Remission



Collagen III in Never Asthma

Table 18: Subepithelial periostin deposition

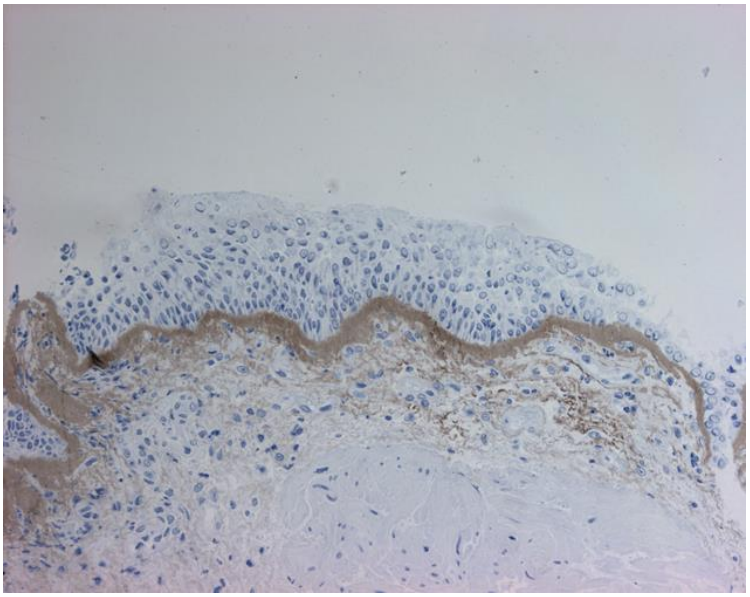


Dot plot of periostin (%) by asthma group (horizontal line indicates median)

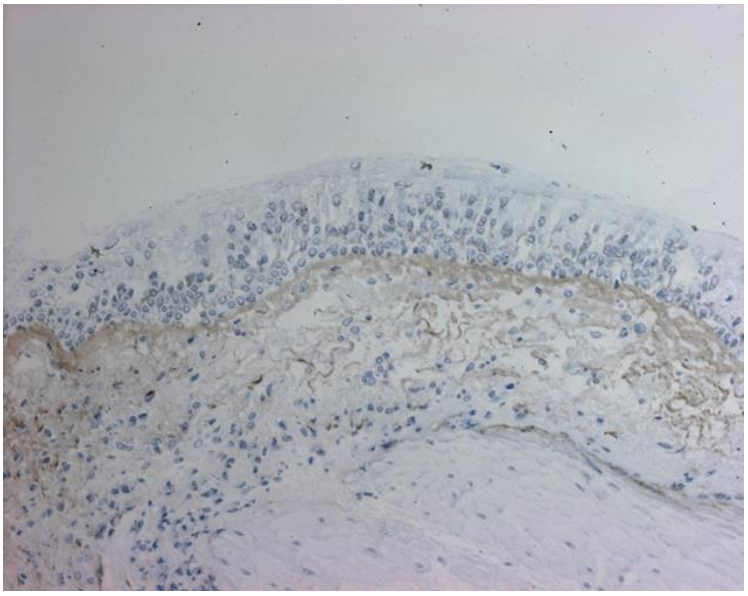
	Median	**IQR**	**n**
Persistent Asthma	39.67	7.38	8
Asthma Remission	31.15	11.53	13
Never Asthma	21.65	6.84	11

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p < 0.001	Reject the Null hypothesis

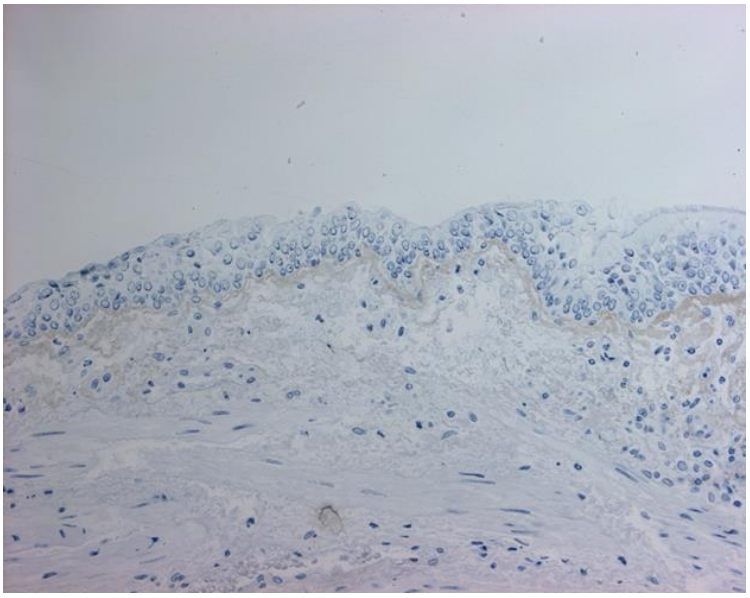
Asthma Persistence vs Asthma Remission	p = 0.025
Asthma Persistence vs Never Asthma	p = 0.002
Never Asthma vs Asthma Remission	p = 0.014



Periostin in Persistent Asthma

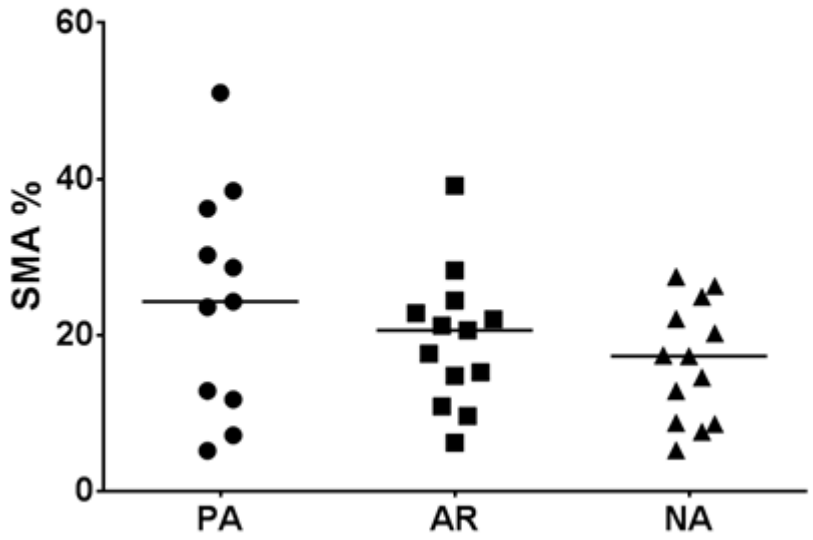


Periostin in Asthma Remission



Periostin in Never Asthma

Table 19: Alpha smooth muscle actin (α SMA)



Dot plot of αSMA % by asthma group (horizontal line indicates median)

	Median	**IQR**	**n**
Persistent Asthma	24.30	24.42	11
Asthma Remission	20.64	10.80	13
Never Asthma	17.32	14.79	13

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.247	Retain the Null hypothesis

Other studies looking at remodelling indices report BM thickness comparable to those reported in this study (table x).

Table 20: BM thickness as reported by other studies	
Kosciuch et al 2012 *(Kosciuch, Krenke et al. 2012)*	Asthmatic subjects 12.54µm +/- 2.8µm
Brewster et al 1990 (Brewster, Howarth et al. 1990)	Asthmatic subjects 3.75µm to 11.1µm Controls 2.16µm to 6.26µm
Broekema et al 2011 (Broekema, Timens et al. 2011)	CoR asthma 6.3µm (4.7-8.4) ClinR asthma 6.5µm (3.8-11.7)

Collagens I and III have been reported at levels of 41.9% (13.9-77.9) in asthmatic subjects, 38.8% (14.5-54.9) in CoR asthma and 36.7 (8.2-64.5) in ClinR asthma by *Broekema et al (Broekema, Timens et al. 2011)*and 62 ± 7% in asthmatic subjects vs 51 ± 9% in control subjects by *Wilson et al (Wilson and Li 1997)* . These are consistent with the findings of this study.

Periostin was analysed in the immediate subepithelial area in this study and this was a novel technique therefore it was not possible to correlate with other current literature.

CHAPTER 6 MARKERS OF EPITHELIAL DAMAGE

6.1 ANALYSIS OF EPITHELIAL EGFR

Epithelial EGFR was measured in 4 subjects with PA, 10 with AR and 9 with NA. Median epithelial EGFR in the PA group was 39.45% (IQR 5.56), in the AR group was 38.71% (IQR 10.17) and in the NA group was 25.67% (IQR 10.39). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of 0.007 between groups (table 20). Further analysis revealed that those with PA and AR had a significantly higher epithelial EGFR than those with NA (p=0.031 and p=0.030 respectively). The difference in epithelial EGFR between those with PA and AR was not significant.

6.2 ANALYSIS OF EPITHELIAL $P21^{WAF}$

Epithelial $p21^{waf}$ was measured in 9 subjects with PA, 11 with AR and 9 with NA. Median epithelial $p21^{waf}$ in the PA group was 32.50% (IQR 8.75), in the AR group 30.76% (IQR 12.85) and in the NA group 22.03% (IQR 12.12) (table 21). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated non-significance between the groups.

6.3 ANALYSIS OF EPITHELIAL IL-8

Epithelial IL-8 was measured in 11 subjects with PA, 13 with AR and 13 with NA. Median epithelial IL-8 in the PA group was 35.89% (IQR 10.63), in the AR group was 30.07% (IQR 10.85) and in the NA group was 28.84% (IQR 9.65). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated a p value of 0.029 between groups (table 22). Further analysis revealed that those with PA had a significantly higher epithelial IL-8 than both those with AR and those with NA (p=0.007 and p=0.005 respectively). The difference in epithelial IL-8 percentage between those with AR and NA was not significant.

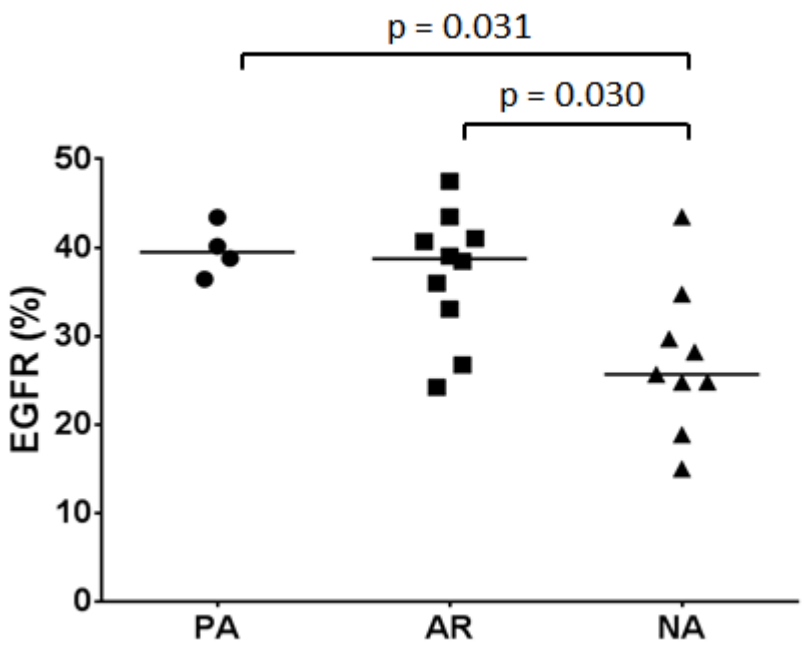
6.4 ANALYSIS OF EPITHELIAL IL-33

Epithelial IL-33 was measured in 8 subjects with PA, 13 with AR and 11 with NA. Median epithelial IL-33 in the PA group was 37.28% (IQR 16.61), in the AR group 30.91% (IQR 22.98) and in the NA group 30.16% (IQR 12.82) (table 23). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated non-significance between the groups .

6.5 ANALYSIS OF EPITHELIAL TGF-BETA$_2$

Epithelial TGF-Beta$_2$ was measured in 2 subjects with PA, 6 with AR and 6 with NA; fewer than the other parameters checked due to problems with the antibody. Median epithelial TGF-Beta$_2$ in the PA group was 47.95% (IQR N/a), in the AR group 29.51% (IQR 13.19) and in the NA group 26.31% (IQR 14.86) (table 24). Non-parametric significance testing using the Kruskal Wallis test was performed and demonstrated non-significance between the groups. However, looking at the box and whisker plot the mean TGF-Beta$_2$ in the PA group show a trend to be higher than in the other two groups, but sample analysis was insufficient to prove significance.

Table 21: Epithelial deposition of EGFR

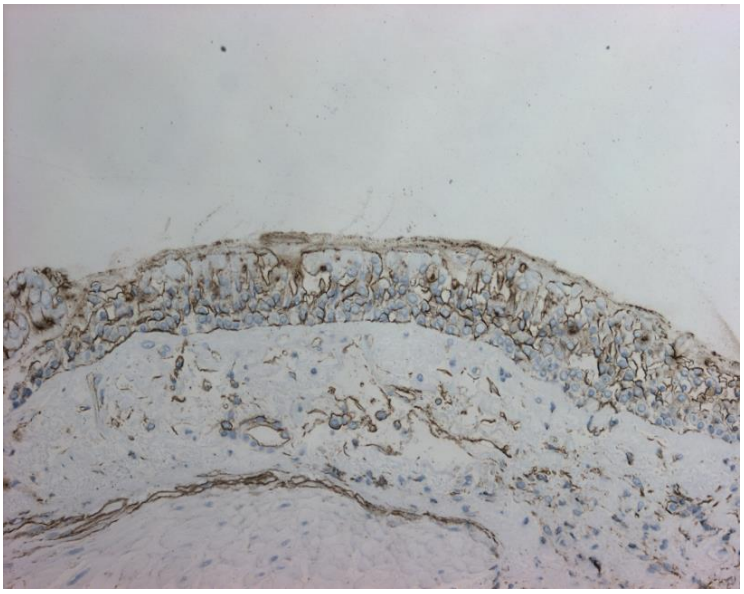


Dot plot of EGFR in epithelium (%) by asthma group (horizontal line indicates median)

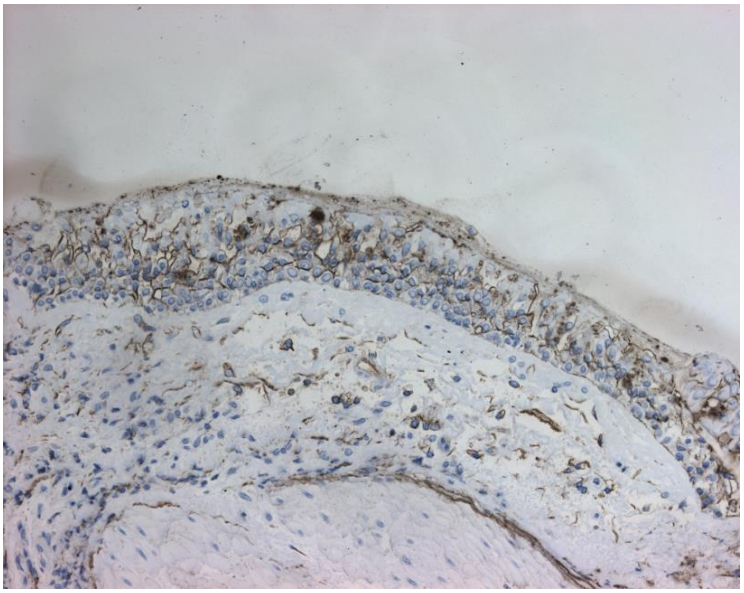
	Median	**IQR**	**n**
Persistent Asthma	39.45	5.56	4
Asthma Remission	38.71	10.17	10
Never Asthma	25.67	10.39	9

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.007	Reject the Null hypothesis

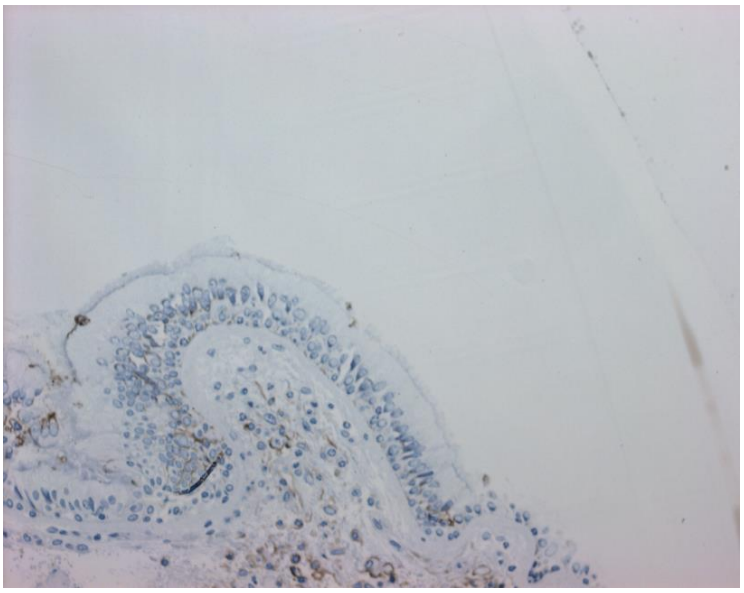
Asthma Persistence vs Asthma Remission	p = 0.671
Asthma Persistence vs Never Asthma	p = 0.031
Never Asthma vs Asthma Remission	p = 0.030



EGFR in epithelium (%) in Persistent Asthma

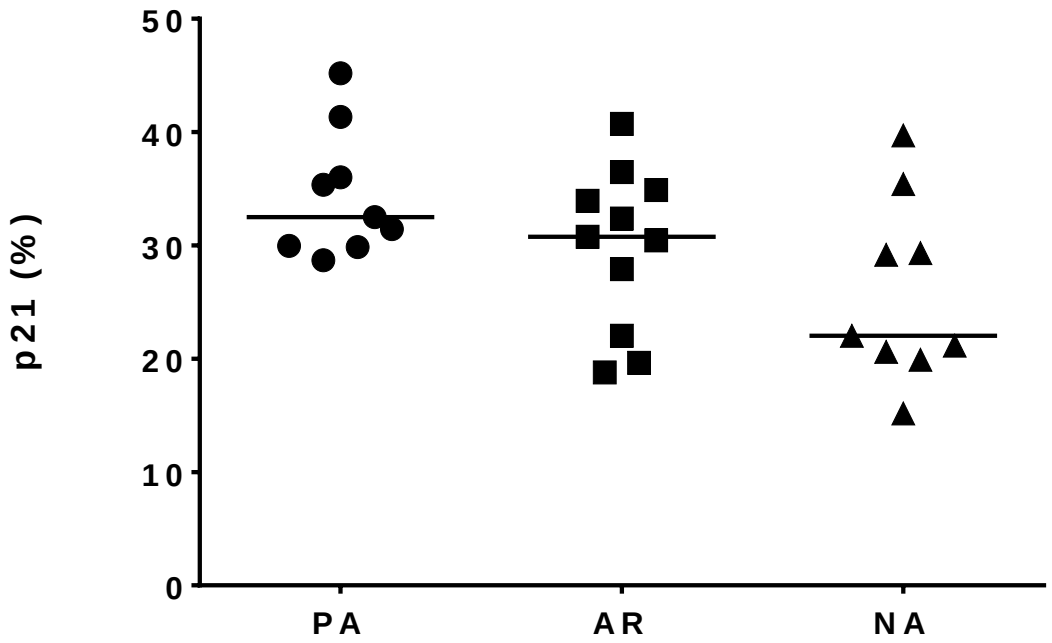


EGFR in epithelium (%) in Asthma Remission



EGFR in epithelium (%) in Never Asthma

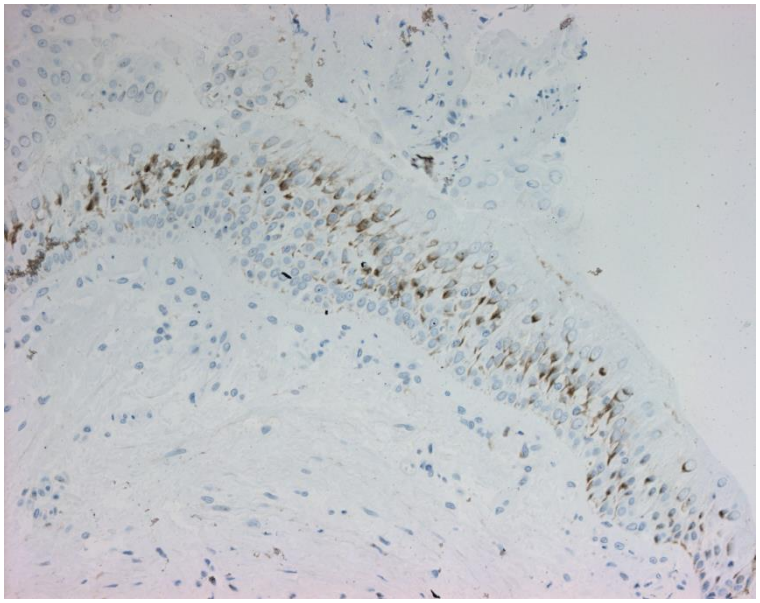
Table 22: Epithelial deposition of p21^{waf}



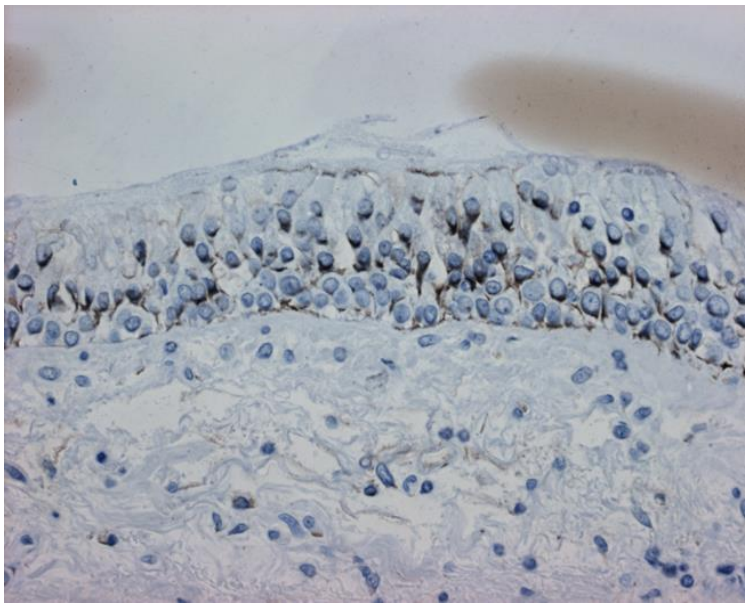
Dot plot of p21in epithelium (%) by asthma group (horizontal line indicates median)

	Median	IQR	n
Persistent Asthma	32.50	8.75	9
Asthma Remission	30.76	12.85	11
Never Asthma	22.03	12.12	9

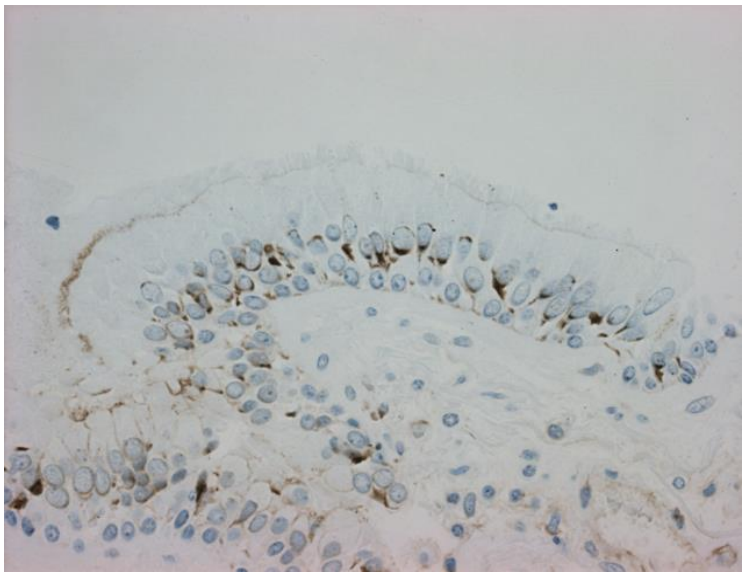
Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.147	Retain the Null hypothesis



p21 in Persistent Asthma

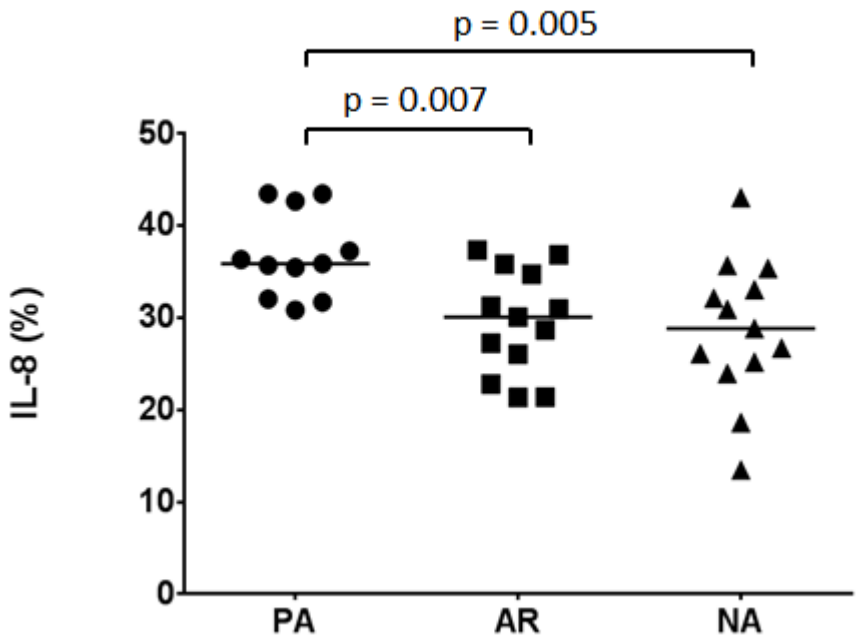


21 in Asthma Remission



p21 in Never Asthma

Table 23: Epithelial deposition of IL-8

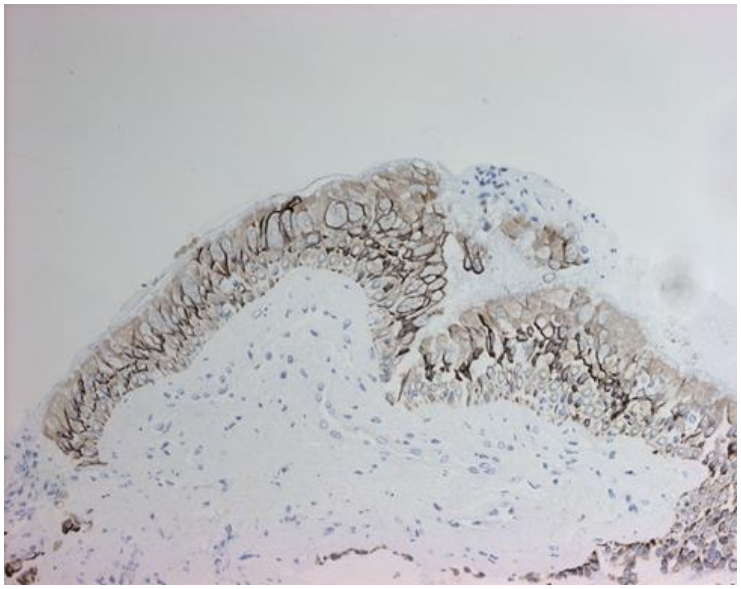


Dot plot of IL-8 in epithelium (%) by asthma group (horizontal line indicates median)

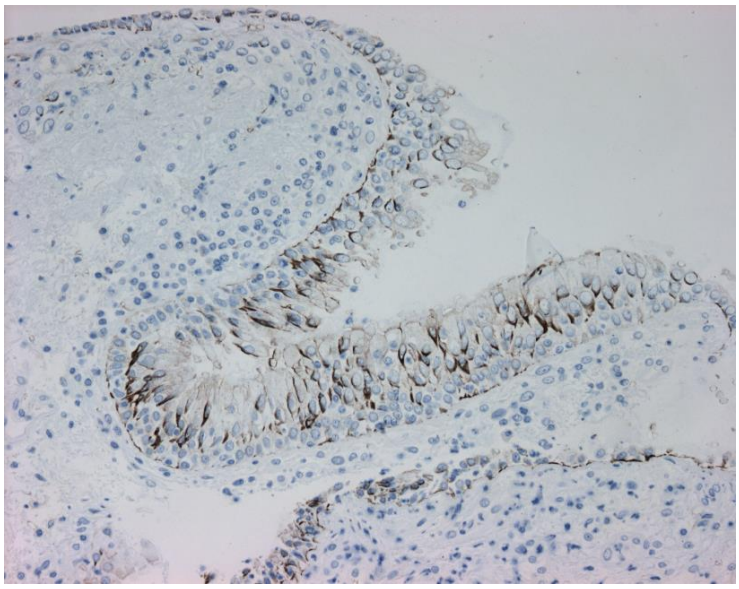
	Median	**IQR**	**n**
Persistent Asthma	35.89	10.63	11
Asthma Remission	30.07	10.85	13
Never Asthma	28.84	9.65	13

Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.029	Reject the Null hypothesis

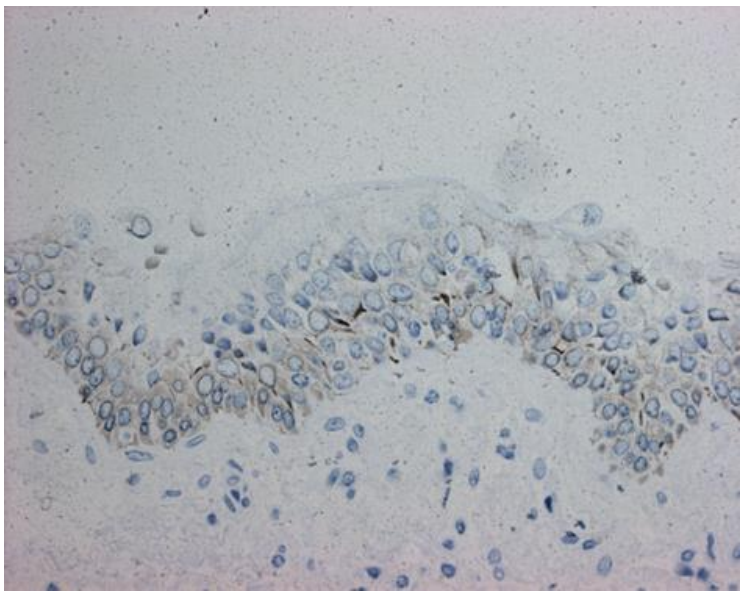
Asthma Persistence vs Asthma Remission	p = 0.007
Asthma Persistence vs Never Asthma	p = 0.005
Never Asthma vs Asthma Remission	p = 0.739



IL-8 in Persistent Asthma

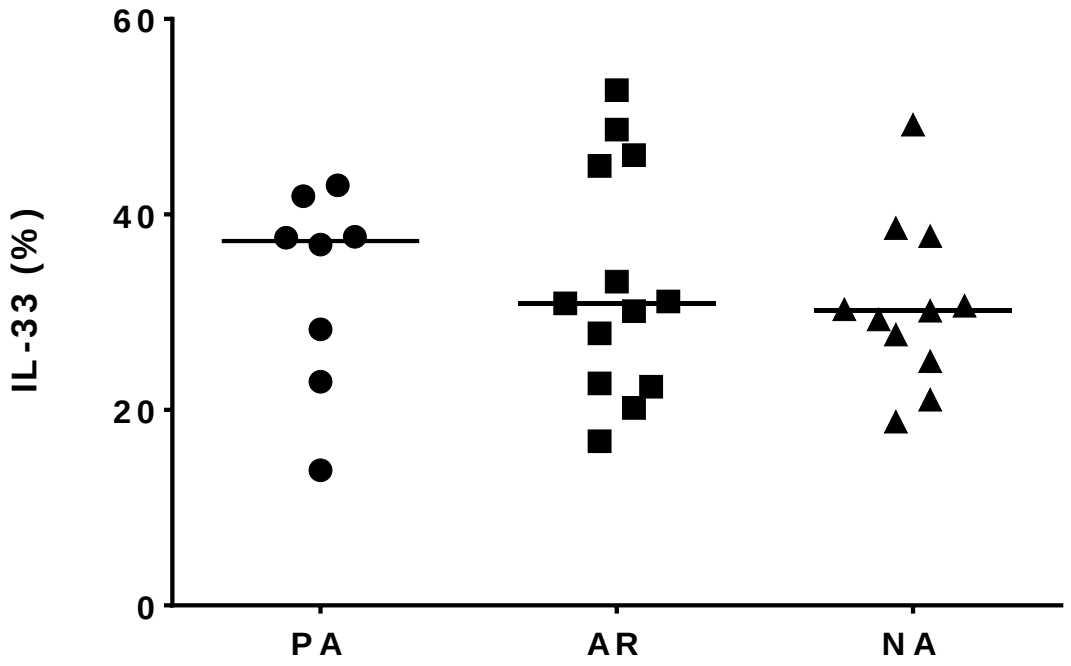


IL-8 in Asthma Remission



IL-8 in Never Asthma

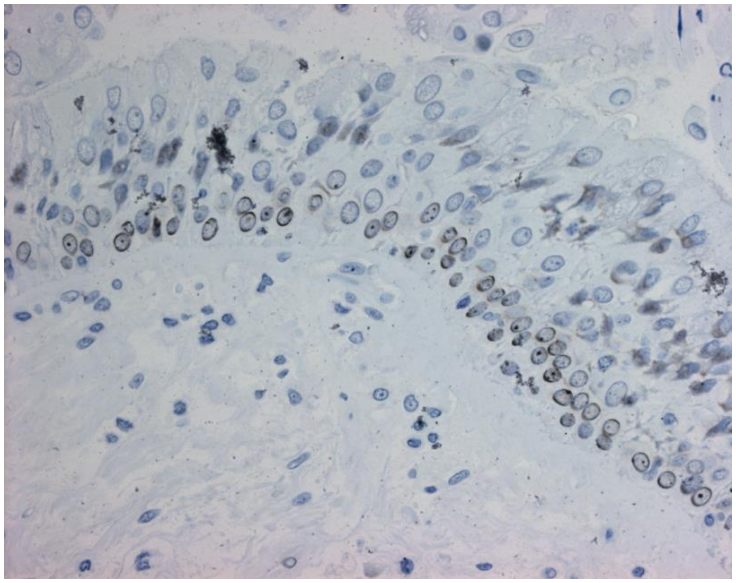
Table 24: Epithelial deposition of IL-33



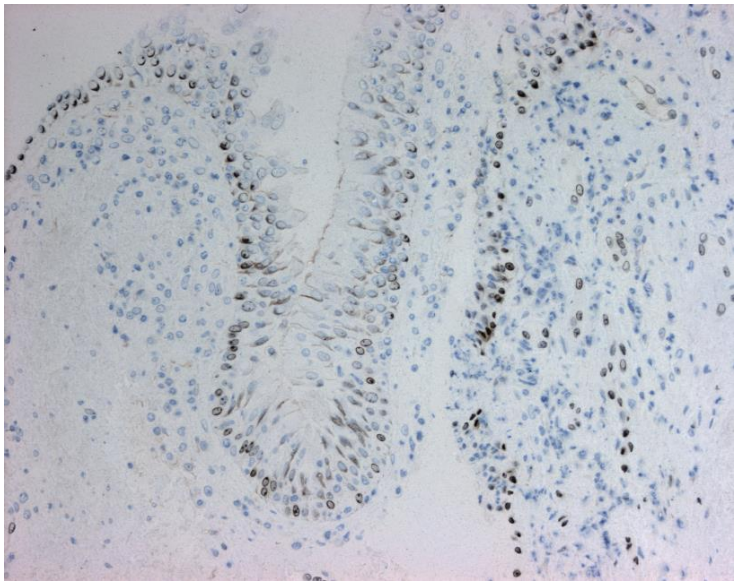
Dot plot of IL-33 in epithelium (%) by asthma group (horizontal line indicates median)

	Median	IQR	n
Persistent Asthma	37.28	16.61	8
Asthma Remission	30.91	22.98	13
Never Asthma	30.16	12.82	11

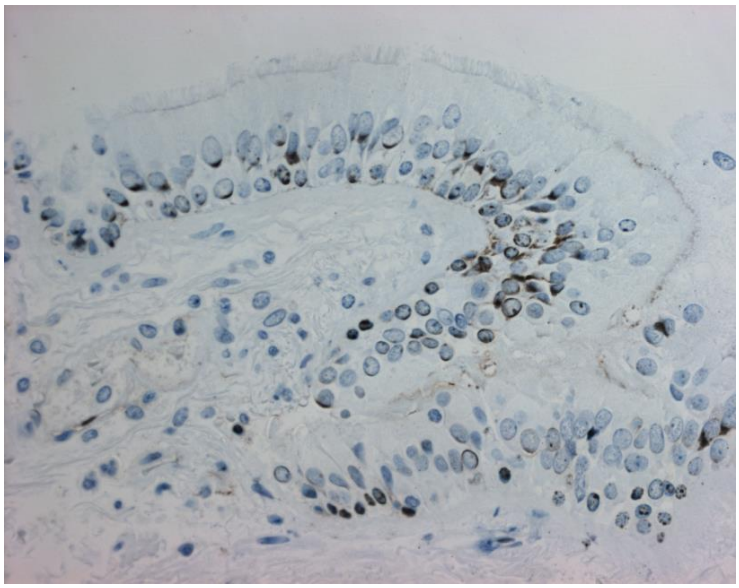
Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.498	Retain the Null hypothesis



IL-33 in Persistent Asthma

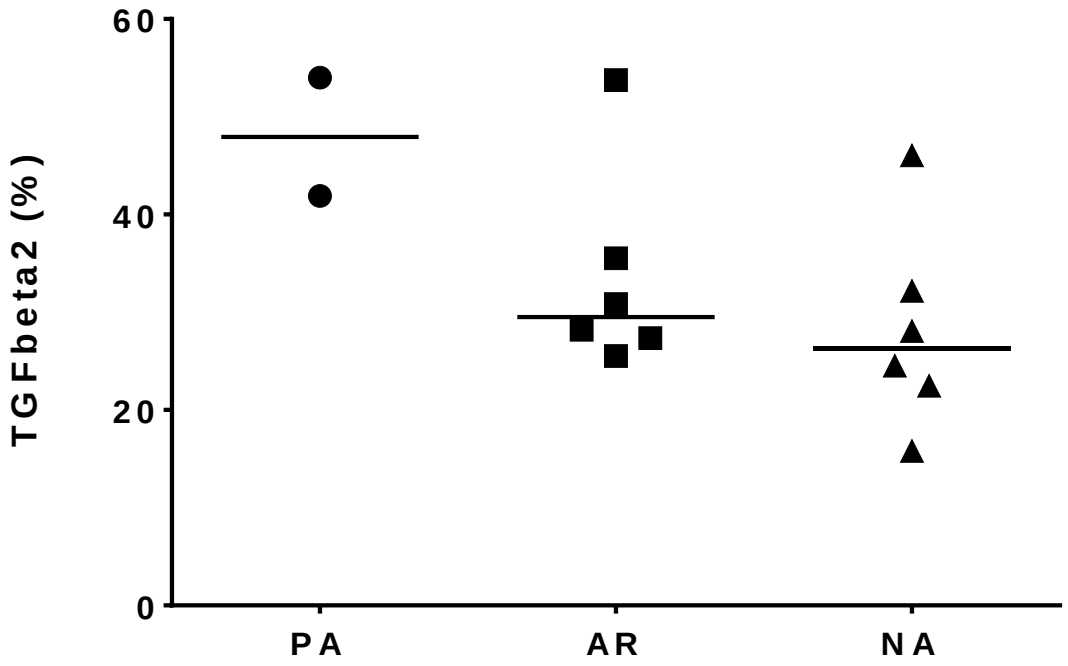


IL-33 in Asthma Remission



IL-33 in Never Asthma

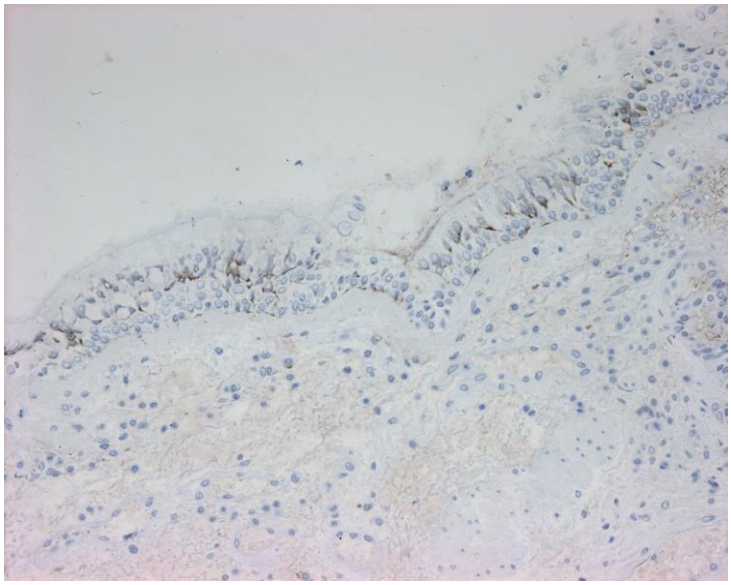
Table 25: Epithelial deposition of TGFβ$_2$



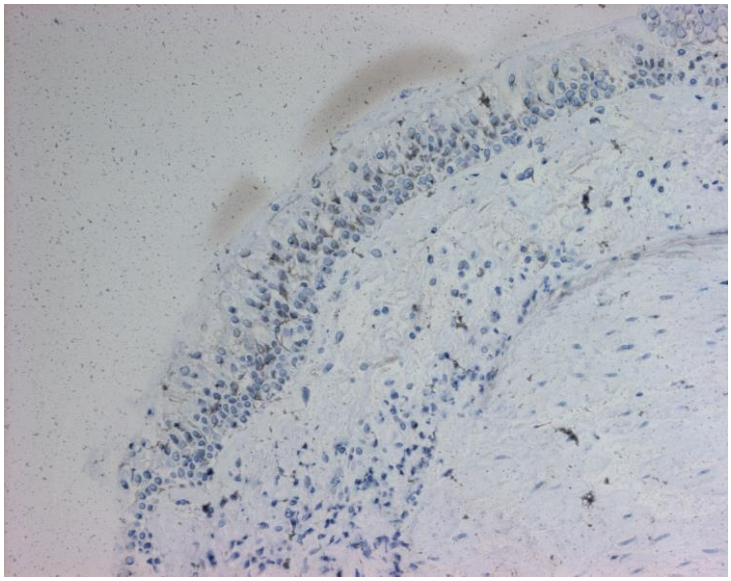
Dot plot of TGFβ$_2$in epithelium (%) by asthma group (horizontal line indicates median)

	Median	IQR	n
Persistent Asthma	47.95	-	2
Asthma Remission	29.51	13.19	6
Never Asthma	26.31	14.86	6

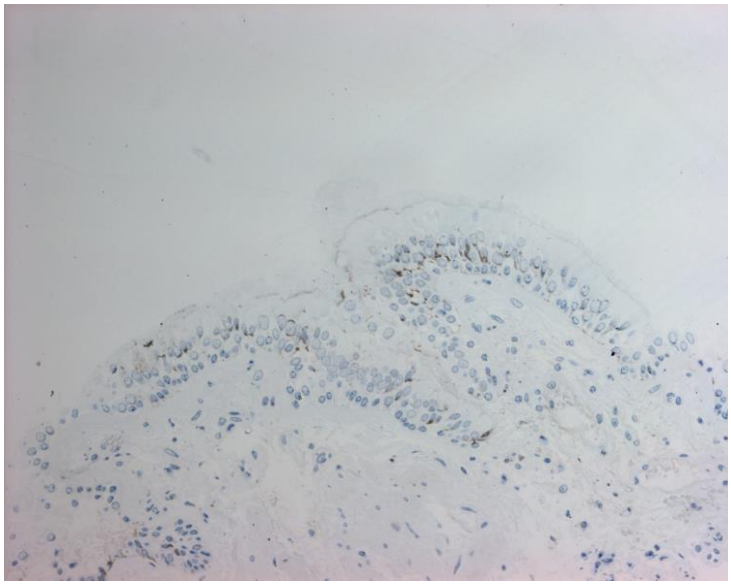
Significance testing (non-parametric)		
Between groups Kruskal Wallis test	p = 0.118	Retain the Null hypothesis



TGFβ_2 in epithelium in Persistent Asthma



TGF$\beta_2$in epithelium in Asthma Remission



TGFβ_2 in epithelium in Never Asthma

CHAPTER 8 DISCUSSION AND CONCLUSION

This is a nested case-control study where individuals were recruited from a well-established birth cohort. Three groups of individuals recruited were; those with PA (n = 16), AR (n = 19) and NA (n = 20) based on their history (presence or absence) and progression of asthma. They were well-matched with regards to height, weight, body mass index, socioeconomic indices and exposure to allergens. Those with PA were more likely to be atopic than those with NA, as would be expected; and those with AR were midway between the other two groups.

It is well recognised that children with asthma can be seen to 'outgrow' their disease and that this occurs commonly in the second decade. However, more recently, several groups, including *Boulet* and co-workers, have described the persistence of pulmonary function abnormalities and (asymptomatic) bronchial hyper-responsiveness in adolescents and young adults who have demonstrated clinical remission of asthma symptoms and who no longer require asthma medications (Boulet, Turcotte et al. 1994). In addition, higher numbers of eosinophils in BAL fluid from individuals with clinical remission of asthma compared with healthy controls. This suggests that there is ongoing inflammation present which does not represent a true remission of asthma and may lead to relapse later in life; in fact the presence of BHR has been demonstrated in epidemiological studies to be a risk factor for future development of asthma symptoms.

These findings have led to a distinction between 'clinical remission of asthma' (ClinR) (defined as "no asthma symptoms or asthma mediation use for a period of time, usually 12-24 month, but evidence of persistent subclinical BHR"), and 'complete remission of asthma' (CoR) (defined as "no asthma symptom or asthma mediation use for a period of time, usually 12-24 months, and no evidence of BHR"). This study, due to its recruiting methods, can be considered as looking exclusively at the CoR group as all the AR patients were required to have absence of symptoms, no use of asthma medications and a negative bronchial challenge with Mannitol.

We demonstrated that those with PA and AR had significantly lower percentage predicted FEV_1 compared to those with NA. Only a small number of participants had airflow

obstruction (defined as an FEV_1/FEV ratio of <70%) all of whom had persistent asthma. Bronchial biopsy revealed that levels of eosinophils were significantly raised in those with PA (compared to NA) as one would expect but those with AR had similarly low levels as those with NA. These findings are similar to those of *Broekema et al* who demonstrated that eosinophil degranulation was the best differentiating factor between those with CuA and those with CoR (Broekema, Timens et al. 2011). In our study neutrophils and mast cell numbers were not significantly different between groups. Work from other groups has supported these findings; levels of neutrophils has been shown to be increased in severe corticosteroid-resistant asthma but not mild to moderate disease (Wenzel, Szefler et al. 1997, Jatakanon, Uasuf et al. 1999) and mast cell activation is related to asthma exacerbation but only one study has demonstrated a correlation between mast cells and BHR,(Koshino, Arai et al. 1995) other groups have failed to correlate mast cells to asthma severity (Bousquet, Chanez et al. 1991, Jarjour, Calhoun et al. 1991).

The above findings support the idea that the inflammatory component of airflow obstruction is not present in those with AR; thus explaining the lack of symptoms in this group. This led to the hypothesis that the lower FEV_1 could be accounted for by persistent changes of remodelling in the AR group in the absence of evidence of persistent inflammation and we examined the bronchial biopsies for evidence to support this hypothesis.

In this study thickness of the lamina reticularis (collagen band thickness) was significantly higher in the AR and PA group than those with NA. This suggests that, despite clinical remission of asthma symptoms and absence of BHR, there is still evidence of remodelling in the AR group confirming our hypothesis. This correlates with work done by *Bossley et al* who demonstrated that remodelling changes occur early in those with childhood asthma (Bossley, Fleming et al. 2012), and this study adds to this knowledge by demonstrating that the remodelling changes persist into early adulthood, despite clinical remission.

Our study also looked at subepithelial deposition of collagens I and III in bronchial biopsies. We demonstrated a similar pattern for both of these markers whereby those with PA had the highest proportion, followed by those with AR then those with NA. This may suggest that without the driving influence of inflammation the deposition of subepithelial collagen

may slow down or even partially remit. An alternative explanation however, might be that those with AR had less severe asthma during childhood and which in turn facilitated remission. The fact that 11 of 16 (68.8%) of the PA group were using ICS at the time of bronchial biopsy needs to be taken into account and may have an influence on the subepithelial collagen levels in this group. The number of participants in the PA group was not sufficient to analyse subgroups.

The findings of increased subepithelial fibrosis in bronchial biopsies taken from asthmatic subjects is well described. There is evidence that increased collagen levels can be found in asthmatics with mild or intermittent disease (Roche, Beasley et al. 1989), in those who have only recently received an asthma diagnosis (Boulet, Turcotte et al. 2000) and evidence of remodelling has been reported in children as young as four years old (Martinez, Wright et al. 1995, Fedorov, Wilson et al. 2005, Turato, Barbato et al. 2008). Several studies have noted the absence of a correlation between subepithelial fibrosis and either the severity of asthma or the duration of symptoms (Martinez, Wright et al. 1995).

They also showed that those with current asthma who were using ICS had the least evidence of BM thickening of all groups; however there were no healthy controls for comparison in this study. From this they inferred that the BM thickness *per se* is not likely to be responsible for clinical bronchoconstriction but instead may be a form of 'residual scarring' from exposure to asthma symptoms for several years.

Taking these findings collectively we know that remodelling can occur early in life after asthma has been present for only a short time and can persist into adult life. If these changes exist during adolescence they appear to have a deleterious effect on lung growth leading to decreased FEV_1 in adulthood. In this study (as described previously) those with PA and AR had similar decrease in FEV_1 compared with subjects with NA.

As a result, strategies to influence remodelling using anti-inflammatory treatments (such as ICS) have been examined; however the results have been contradictory. *Hoshino et al* were able to demonstrate a decrease in subepithelial collagen III deposition after six months of using high dose ICS compared with placebo (Hoshino, Takahashi et al. 1999). *Sont et al* examined an approach whereby BHR rather than symptoms was targeted with ICS and demonstrated a decrease in subepithelial reticular collagen after 2 years of

treatment (Sont, Willems et al. 1998). Most studies, however, required the use of very high doses of ICS at a level that would be above the recommended dose for most people with asthma and a long treatment period. Only one study by *Olivieri et al* demonstrated decrease in markers of remodelling with low-dose inhaled fluticasone over a shorted period of time (Olivieri, Chetta et al. 1997). Studies using lower doses or shorter treatment periods tended to show no significant change on subepithelial collagen (Jeffery, Godfrey et al. 1992). This supports the view that prolonged high dose ICS use may reduce changes associated with remodelling.

In light of these findings, should children who are being treated for asthma continue with ICS, despite lack of symptoms and BHR, until they achieve full lung growth in order to minimise the amount of remodelling of the BM and sub-epithelial area? This has not previously been explored but the high doses of ICS that seem to be required may mean that in practice this is not achievable and adverse effects of high-dose ICS may be unacceptable.

A recent article by *Grainge et al* looked at the effect of bronchoconstriction on remodelling in asthmatic subjects and found that bronchoconstriction induced remodelling independent of inflammation (Grainge, Lau et al. 2011). Considering the fact that those with AR have some degree of bronchoconstriction (not enough to qualify them for a positive Mannitol challenge test but a significantly larger drop in FEV_1 than those with NA) it is possible that they could have ongoing subclinical bronchoconstriction not detected by Mannitol provocation testing (which primarily acts via inflammatory mediators) which is contributing to the ongoing changes of remodelling. An article about the role of mechanical forces in remodelling by *Manuyakorn* (Manuyakorn 2014) examines the possibility of such a theory; drawing on work done by *Bossley et al* (Bossley, Fleming et al. 2012) that demonstrates early remodelling changes in young children with severe asthma in the absence of significant inflammation. This leads us to the possibility that the use of ICS as maintenance therapy does not address potential subclinical bronchoconstriction that could be contributing to remodelling independent of inflammation. This may suggest that use of

LABAs alongside ICS would be an effective way to target both inflammation and bronchoconstriction in order to minimise remodelling changes that occur.

This study has allowed us to further characterise the remodelling changes that occur in young adults who have had asthma since childhood and also to look at the remodelling seen in young adults who have grown out of childhood asthma.

Periostin deposition in bronchial biopsies was analysed as a novel marker of remodelling. Periostin is a matricellular protein associated with fibrosis and remodelling in the ECM of asthmatic subjects (Takayama, Arima et al. 2006); it is secreted basally into the ECM from BECs and therefore it has been hypothesised that serum periostin levels may be used as a non-invasive markers of airway inflammation (Woodruff, Boushey et al. 2007). Peripheral blood levels of periostin have been shown to be a more reliable indicator of airway eosinophilic inflammation than blood eosinophil levels or fractional exhaled nitric oxide (FENO) (Jia, Erickson et al. 2012). It is therefore used as a surrogate marker of T_H2 inflammation in identifying patients for novel asthma therapies such as Lebrikizumab, a monoclonal antibody against IL-13.

In this study we demonstrated that periostin deposition followed a pattern very similar to subepithelial collagen III and I, being highest in the PA group followed by the AR group then the NA group. The high levels of periostin in subjects with PA and low levels in those with NA is in keeping with the findings described by *Takayama et al* who demonstrated co-localisation of periostin to the subepithelial region with other factors associated with fibrosis such as fibronectin, tenascin C and collagen V in patients with asthma (Takayama, Arima et al. 2006). We also showed correlation between high levels of airway mucosal periostin and eosinophils in the PA group similar to the findings of *Jia et al* and low levels of periostin and eosinophils in the NA group (Jia, Erickson et al. 2012).

Asthmatic subjects with AR have not previously been analysed for periostin deposition and our AR group who had little evidence of inflammation and low levels of eosinophils had a significantly higher level of periostin than those with NA, although not to the extent of those with PA. This would appear to suggest the fact that while periostin is no longer being actively secreted as part of the T_H2 inflammatory process, its presence is still significantly detectable in the ECM. The lower level of periostin in the AR group than the PA group could

either represent the fact that periostin levels in the ECM decrease with time if the T_H2 inflammatory stimulus is not present, or that they remain static but the PA group suffer ongoing periostin deposition due to ongoing inflammation.

Local activation of TGF-β_2 leads to periostin expression in the wound repair scenario and TGF-β has been shown to induce periostin *in vitro* in cells such as smooth muscle and fibroblasts (Frangogiannis 2012). This study has demonstrated low levels of TGF-B_2 in those with NA and AR indicating that active collagen deposition may not be occurring in these groups. This is consistent with the observation that higher levels of TGF-B_2 (not reaching statistical significance) are detected in the PA group as they have ongoing inflammation, and remodelling remains an active process.

EGFR and $p21^{waf}$ are key factors in repair of the bronchial epithelium. We demonstrated in this study that those with NA had low levels of epithelial EGFR and those with PA had high levels of epithelial EGFR, which would be expected from the current literature (Amishima, Munakata et al. 1998, Puddicombe, Polosa et al. 2000). The AR group also had high levels of EGFR, similar to those with PA. This, coupled with low levels of inhibitory factor $p21^{waf}$, might suggest that the EGFR have remained upregulated in order to repair damage to the epithelium, which it is now able to do, in the absence of the inhibitory influence of $p21^{waf}$. Analysis of $p21^{waf}$ in this study showed a trend for PA to have higher levels than AR and NA, however statistical significance was lacking.

This study demonstrated that epithelial IL-8 levels were higher in the PA group and low in those with AR and NA. This correlates with the stud by *Marini et al* who demonstrated increased IL-8 and IL-8 mRNA levels in BEC isolated from asthmatic vs control subjects (Marini, Vittori et al. 1992). Other researchers have suggested a link between high IL-8 levels and systemic inflammation (Silvestri, Bontempelli et al. 2006, Maneechotesuwan, Essilfie-Quaye et al. 2007); however they were measuring IL-8 in sputum and serum.

Levels of epithelial IL-33 were not significantly different between the three groups, this may be related to study methods and design rather than representing lack of true difference. IL-33 was measured as percentage nucleolar staining which differed from the methods used to assess other parameters and which was technically more difficult. IL-33 has been shown in other studies to be expressed in higher levels in bronchial biopsies from subjects

with allergic asthma than healthy controls.(Kurowska-Stolarska, Stolarski et al. 2009) As a T_H2 mediator we would have expected it to be higher in the PA group than those with NA in correlation with TGF-B_2, periostin and eosinophils.

These findings suggest that the group with PA have active evidence of ongoing epithelial damage whereas those with AR and NA do not, which is what would be expected in the absence of an inflammatory response driving the process. This correlates with the findings of low eosinophil levels and lack of BHR in the AR group.

Historically all indices of remodelling have been thought to be related to BHR and lower lung function; however this may not in fact be the case. In 2010 *Broekema et al* looked at a group of 164 adults (mean age 49 years) who had current asthma (CuA), ClinR and CoR and examined bronchial biopsies for evidence of remodelling and inflammation. Some of their findings correlate with those of this study despite the difference in age of cohort. They demonstrated that basement membrane thickness was comparable between those with clinical remission, complete remission and current asthma (Broekema, Timens et al. 2011). They suggest that BM thickness is therefore not related to BHR but could be a form of scarring in the lung from previous disease state. This study's findings correlates with that by demonstrating a significantly thickened BM in individuals with asthma remission who do not clinically express BHR. BM thickness may contribute to the lower pre-bronchodilator FEV_1 seen in those with PA and AR.

Collagen deposition within the subepithelial extracellular matrix seems to better reflect the clinical scenario as those with AR demonstrate an 'intermediate' position between those with PA and AR. This is also seen with using Periostin as a marker of remodelling. This fibrosis of the ECM may represent an element of the remodelling process that could be linked to asthma remission.

8.1 STRENGTHS AND LIMITATIONS

Strengths of this study include the fact that participants were recruited from a whole population cohort rather than from a hospital-based setting and therefore are more likely to be representative of the asthmatic population in the community. We also had available to us information collected throughout the cohort follow-up from birth and therefore information regarding age of asthma onset or never having wheezed etc. could be validated, if necessary, which reduced recall bias. There was also no researcher bias in selecting the subgroup to be analysed from the main cohort as this was determined by the responses of the participants. This may, however, have introduce some bias as they were a self-selected sample.

Limitations included the lack of more severe asthmatics (BTS stage 4-5) involved in the study. For most comparisons, this would only have served to augment the most significant differences found with regards to remodelling factors; however it may have had an influence on analysis of epithelial factors, several of which failed to show any significant differences between groups. In addition, several of the cell-counting parameters, particularly eosinophils, involved only a small number of samples due to difficulty with the technique and time limitations. There were several 'outlying' values which may have affected interpretation of the data.

8.2 CONCLUSION

This study demonstrates that young adults who have achieved clinical and pathophysiological remission of childhood asthma still have significant differences to those who have never had asthma. Their bronchial biopsies demonstrate persistent evidence of remodelling and fibrosis in the form of collagens and periostin in the ECM and thickening of the basement membrane with collagen, despite absence of evidence of active inflammation. This suggests that remodelling changes occur early and persists after the disease process has been quiescent for some time. Moreover, AR group had lower lung function (compared to NA) despite absence of symptoms, inflammation or BHR, indicating that remodelling may have long term consequences on lung function, which may be particularly important during adolescence as a period of rapid height and lung growth. Given some evidence that anti-inflammatory asthma therapies can reduce ECM fibrosis, one might suggest that asthma in children should be more aggressively treated to prevent remodelling changes. Moreover, children who 'grow out' of asthma should be closely monitored during their adolescent years to ensure that they are achieving healthy lung growth. A case could be made to continue ICS therapy in adolescents despite loss of symptoms or lack of inflammation but this would have to be carefully weighed against the side effects of ICS.

We do not currently know how to predict the long term outcome of those who grow out of asthma as children. We know that some of them relapse in later life but are not able to identify them, as the characteristics of those who will relapse, apart from severity, are not known. Inevitably some of these individuals will be smokers; are they at higher risk of developing chronic obstructive pulmonary disease (COPD) as middle-aged adults due to their persistent remodelling changes? Further research is necessary to determine what happens to young adults who have entered asthma remission during adolescence and how they should be monitored and managed.

The Isle of Wight Birth Cohort is well-placed as an established longitudinal study to take this research forward. The cohort will be studied again aged 26 years and characterised for asthma remission or persistence as they progress through their third decade, they will also be examined for early evidence of COPD, in particular those with asthma who had started smoking. This will further enable us to look at the effects of smoking on these groups and evidence of an asthma/COPD overlap syndrome

APPENDICES AND ACCOMPANYING MATERIALS

APPENDIX 1: Study Personnel

Members who participated in the EMTU study are listed below:

Research Fellow:	Dr Sian Evans (SE)
Cohort Study Supervisor:	Professor Hasan Arshad (SHA)
Research/Bronchoscopy Nurses:	Mrs Frances Mitchell (FM) Mrs Sandra Pink (SP) Mrs Laura Presland (LP) Mrs Caroline Smith (CS) Mrs Shuna Egerton (SE)
Administration:	Mr Stephen Potter (SP) Mrs Sharon Matthews (SM)
Laboratory advisors and sample collecting:	Mrs Susan Wilson (SW) Mr Jon Ward (JW) Ms Helen Rigden (HR) Ms Jenny Norman (JN)
Laboratory cytokine analysis:	Dr Laurie Lau (LL)

Appendix 2: Accompanying Materials Used

Participant Invitation Letter - First Visit

Bronchoscopy Invitation Letter

Letter to GP

Patient Information Sheet

Consent Form

ISAAC Written Questionnaire

Mannitol Bronchial Challenge Report Form

Case Report Form (CRF)

University of Southampton School of Medicine

Isle of Wight NHS Primary Care Trust

Southampton NHS University Hospitals NHS Trust

MRC EMTU-study 2

THE DAVID HIDE ASTHMA AND ALLERGY RESEARCH CENTRE

St Mary's Hospital, Newport, Isle of Wight, PO30 5TG

Tel: +44(0)1983 534898 Fax: +44(0)1983 822928 Study e-mail: sle1r10@soton.ac.uk

Date xx/xx/xx

Dear participant name

An appointment has been made for you to attend the David Hide Asthma and Allergy Centre at St Mary's Hospital, IOW on xx/xx/xx at xxam/pm. This visit will take approximately 2 hours. This will consist of a skin prick test, questionnaires, spirometry, providing a urine sample and a bronchial challenge test.

Before this visit please do not drink or eat any caffeine-containing products (e.g. coffee, tea, coke, chocolate) for 4 hours. Please do not perform any heavy exercise on the day of the visit.

If you take asthma medication please withhold your medications for the time specified in the table below. If you are not sure about any medications (or they are not included in the table) please ask one of the research team for advice. Should you become wheezy/tight and need to take your inhalers during this time please do so and contact us to let us know.

Time to withhold	Medication
6-8 hours	**Inhaled non-steroidal anti-inflammatory agents** e.g. sodium cromoglycate, necrodomil sodium
8 hours	**Short acting beta-2 agonists** e.g. salbutamol (Ventolin), terbutaline (Bricanyl)
12 hours	**Inhaled corticosteroids** e.g. beclamethasone (Qvar, Clenil, Becotide), budesonide (Pulmicort), fluticasone (Flixotide)
12 hours	**Ipratropium bromide** (Atrovent)
24 hours	**Long-acting beta-agonists** e.g. salmeterol (Serevent), formoterol (Oxis) *and* **Combination inhalers containing the above** e.g. Seretide, Symbicort, Fostair
24 hours	**Theophylline tablets** e.g. Uniphyllin, Nuelin, Slo-phyllin
72 hours	**Tiotropium bromide** (Spiriva)
72 hours	**Anti-histamine tablets** e.g. cetirizine, fexofenadine, loratidine
4 days	**Leukotriene receptor antagonists** e.g. montelukast (Singulair), zafirlukast

We look forward to seeing you.

With best wishes from the Research Team.

University of Southampton School of Medicine

Isle of Wight NHS Primary Care Trust

Southampton NHS University Hospitals NHS Trust

MRC EMTU-study 2

THE DAVID HIDE ASTHMA AND ALLERGY RESEARCH CENTRE

St Mary's Hospital, Newport, Isle of Wight, PO30 5TG
Tel: +44(0)1983 534898 Fax: +44(0)1983 822928 Study e-mail: sle1r10@soton.ac.uk

Date 19/09/12

Dear participant name

An appointment has been made for you to attend the Wellcome Trust Clinical research Facility at Southampton General Hospital on 11/10/12 at 1pm for a bronchoscopy (camera test). This letter is just a reminder of the preparation necessary before the visit and to give you some information about travelling here.

Preparation

1. You must not have eaten anything for 4 hours before your appointment time. You can have sips of clear fluids up to 2 hours before. If you need to take essential medication during this time please discuss this with a member of the research team (see contact details below).
2. If you take blood thinners (Warfarin, aspirin, clopidogrel) you must make the research team aware of this.
3. If you are diabetic you must make the research team aware of this.
4. You will be given a sedating medication during the procedure and therefore **must** be accompanied home by a responsible adult. They should stay with you for the rest of the day.
5. Our research nurses are more than happy to escort you across from the Isle of Wight to Southampton and back. If you would like them to do this please contact us and let us know as soon as possible so that we can make the necessary arrangements.
6. You should not drive, operate heavy machinery, drink alcohol or work for the rest of the day.

Directions from the Isle of Wight

The easiest way to get to the hospital from the Red Jet/Red Funnel is by taxi. There is a taxi rank outside the Red Jet Terminal. Telephone numbers for some other taxi companies are listed below:

Radio taxis: 02380 666666
West Quay cabs: 0700 0700 0700
Aerotaxi: 02380 010203

Alternatively by bus, catch the free blue "City Link" bus from outside the Red Jet/Hythe Ferry terminal entrance to the train station. Cross over the railway line via the footbridge and catch the number 10 bus direct to Southampton General Hospital.

At the hospital

The Wellcome Trust is on level C in the West Wing. As you come in through the Main Entrance turn right at Burger King, walk past the lifts on your right, past the long corridor (sign-posted South Academic block) on your right into the West wing and the Wellcome Trust is on your left. Please report to the reception. There is a map overpage.

If you get lost please call 02380 794989 and one of the team will come to meet you.

We look forward to seeing you in Southampton.

Best wishes from Sian and The Isle of Wight Research Team

Southampton General Hospital

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton
School of Medicine

THE DAVID HIDE ASTHMA AND ALLERGY RESEARCH CENTRE
St Mary's Hospital, Newport, Isle of Wight, PO30 5TG
Tel: +44(0)1983 534898 Fax: +44(0)1983 822928 e-mail: IOWStudy@iow.nhs.uk
Dr Hasan Arshad, Dr Graham Roberts: Research Investigators Mrs Sharon Matthews: Research Nurse

April 2012
Dr *GP Name*
Address

Dear Dr *GP Name*

Re ***Participant name (date of birth)***
Address

We are writing to inform you that your patient is part of a cohort recruited on the Isle of Wight at birth and has consented to participate in a study called "Isle Of Wight Cohort – Bronchoscopy Study". This is based on the Isle of Wight 1989/ 1990 Birth Cohort and may include participants born in 1990/1991 recruited for the house dust mite avoidance study. The study aims to understand how damage and healing of the airways of asthmatics may determine whether asthma will remain throughout adolescence or reoccur in adulthood. This study firstly involves a 3-hour visit to the David hide Asthma and Allergy Centre on the Isle of Wight during which we will undertake completion of questionnaires, skin prick testing, spirometry, exhaled nitric oxide measurements and a mannitol challenge test. At a second 4-hour visit to Southampton General Hospital we will carry out a bronchoscopy. All samples and data will be anonymised prior to analysis to maintain patient confidentiality.

If you have any questions about the study, you are welcome to contact Sharon Matthews or Prof Hasan Arshad at The David Hide Asthma and Allergy Centre, St Mary's Hospital, Newport, Isle of Wight; telephone: 01983 534897; or email: IOWstudy@iow.nhs.uk.

Best wishes,

Yours sincerely

Sharon Matthews	**Hasan Arshad**	**Graham Roberts**
Research Sister	**Professor / Consultant**	**Reader / Consultant**

Patient Information Sheet

ACTIVATION OF THE EPITHELIAL MESENCHYMAL TROPHIC UNIT (EMTU) AND PERSISTENCE OF ASTHMA DURING CHILDHOOD, ADOLESCENCE AND EARLY ADULTHOOD

ISLE OF WIGHT COHORT – BRONCHOSCOPY STUDY

April 2011

INTRODUCTION

We would like to thank you for your involvement so far in the 1989/1990 birth cohort. The data we have been able to collect from you has been helpful in advancing our understanding of asthma and other allergic diseases.

You are now being invited for a follow-up as part of the 1989/1990 birth cohort study. Before you decide to participate it is important for you to understand why the research is being done and what it will involve.

- **Part 1** tells you the purpose of this study and what will happen to you if you take part.
- **Part 2** gives you more detailed information about the conduct of the study.
- **Appendix 1** provides more information for female participants taking part in this study

If anything is not clear and you require more information before you decide whether or not to take part in the study, please telephone the study team on 01983 534897. Additionally, you may find it useful to visit the INVOLVE website (http://www.invo.org.uk/) which has information for patients about research in the NHS.

If you are satisfied with the answers to any questions you may have and would like to take part in the study you will be asked to sign a consent form.

PART 1

1. WHAT IS THE PURPOSE OF THE STUDY?

Over the last few decades there has been a significant increase in the number of teenagers with asthma, eczema, hay fever and food allergy. We still do not know why people develop these diseases. The purpose of this study is to understand how changes in asthma and other allergic diseases relate to the other changes that occur during adolescence. We know that many teenagers outgrow their asthma and allergies but we do not know why this happens. One idea is that damage and healing of the airways of asthmatics may determine whether asthma will remain throughout adolescence or reoccur in adulthood. If we can understand this, we will be able to predict who will outgrow their asthma and it may also provide us with new ideas for treating or even curing asthma and allergic disease.

2. WHY HAVE I BEEN INVITED TO PARTICIPATE?

You have been invited to participate because you are part of the Isle of Wight 1989/1990 birth cohort. This is a birth cohort of nearly one-and-a-half-thousand young people all born on the Isle of Wight in 1989 and 1990. We are asking four groups of 15 young adults to take part. The four groups will include a group who have outgrown their asthma, a group who have asthma now but did not have asthma at age 10, a group that have asthma now and as children and a 'control' group who have never had asthma. Even if you no longer live on the Isle of Wight you may still be eligible to take part.

3. DO I HAVE TO TAKE PART?

Participation in this study is entirely voluntary. It is up to you to decide whether or not to take part. If you do take part, you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw from the study at any time and without giving a reason.

The information sheet and consent form for the study have been approved by an Ethics Committee (EC). The EC is a group of scientific and non-scientific people who safeguard the rights, safety, dignity and well-being of people participating in research.

4. WHAT WILL HAPPEN TO ME IF I TAKE PART?

If you decide that you want to take part in this research study, we will ask you to participate in a variety of tests. The study involves 2 visits. The first to the David Hide Research Centre and the second to the Wellcome Trust Clinical Research Facility at Southampton General Hospital. Both visits require a small amount of preparation and you will have an opportunity to meet a member of the research team before hand to ask any questions or clarify any concerns.

Visit 1 – David Hide Research Centre (approximately 2 – 3 Hours)

We will arrange for you to visit the David Hide Research Centre at a convenient time. Before this visit we will ask you not to drink or eat any caffeine-containing products (e.g. coffee, tea, coke, chocolate) for 4 hours. If you take asthma medication please withhold your medications for the time specified in the table below. If you are not sure about any medications (or they are not included in the table) please ask one of the research team for advice.

Time to withhold	Medication
6-8 hours	**Inhaled non-steroidal anti-inflammatory agents** e.g. sodium cromoglycate, nedocromil sodium
8 hours	**Short acting beta-2 agonists** e.g. salbutamol (Ventolin), terbutaline (Bricanyl)
12 hours	**Inhaled corticosteroids** e.g. beclamethasone (Qvar, Clenil, Becotide), budesonide (Pulmicort), fluticasone (Flixotide)
12 hours	**Ipratropium bromide** (Atrovent)
24 hours	**Long-acting beta-agonists** e.g. salmeterol (Serevent), formoterol (Oxis) *and* **Combination inhalers containing the above** e.g. Seretide, Symbicort, Fostair
24 hours	**Theophylline tablets** e.g. Uniphyllin, Nuelin, Slo-phyllin
72 hours	**Tiotropium bromide** (Spiriva)
72 hours	**Anti-histamine tablets** e.g. cetirizine, fexofenadine, loratidine
4 days	**Leukotriene receptor antagonists** e.g. montelukast (Singulair), zafirlukast

If you are unwell (wheezy/tight/coughing) please take your inhalers as usual and contact us by telephone for advice. Please make sure you bring your inhalers and other medications with you. If you have been unwell or had a respiratory infection in the previous 2 weeks, or are taking oral steroids, please telephone us and we will rebook your visit at a time when you are well and is convenient for you.
Should you be unable to tolerate withholding your medications for the time specified it may still be possible for you to continue in the trial.

We will repeat many of the procedures that you may have been asked to perform at previous visits. Even if you have not visited us before we would like you to participate this time by undertaking all or part of the following:

- Questionnaire about your current health and the medications that you take
- Weight, height, blood pressure and heart rate and check whether you have signs of eczema or asthma

- *Exhaled Nitric Oxide* - Nitric oxide is a gas present in everyone's breath. You will be asked to exhale into a mouthpiece, breathing out at different speeds. By doing this the nitric oxide content of your breath will be measured by a computer. This will measure the inflammation in your airways.
- *Spirometry* – It is a test to measure the amount of air you have in your lungs by breathing in and how well you can push the air back out by blowing it hard into a tube. If your asthma diagnosis has not been confirmed within 12 months of this visit we will repeat the spirometry ('reversibility' test) before and after you inhale salbutamol. This is to measure how much your airways respond to salbutamol. Spirometry may cause you to cough or make your chest feel tight. Salbutamol will be available if this occurs. Salbutamol relaxes muscles in the air passages of the lungs. It helps to keep the airways open, making it easier to breathe. Salbutamol may make you feel a little shaky and increase your heart rate.
- *Lung challenge test* - before we carry out this test we will ask women of child bearing age to supply a sample of urine to confirm that they are not pregnant. (Please read **Appendix 1 – Female participants**). You will be asked to inhale different concentrations of a substance called Mannitol. The substance will be inhaled through a special device with a mouthpiece. Before the test begins and after each period of inhalation you will be asked to blow forcefully into a spirometer. This test is used to determine how responsive (or irritable) your airways are and to determine the severity of any asthma. The inhalation of aerosols may be associated with mild shortness of breath, cough, chest tightness, wheezing, chest soreness or headache. Many patients do not have any symptoms at all. Symptoms (if they occur) are mild, last only a few minutes, and disappear following the inhalation of a bronchodilator medication such as salbutamol.
- *Skin allergy tests* – drops of 13 common allergens will be placed on the skin and scratched to see what you are allergic to. Your arm might itch or become red at the site where the test was done and there may be mild discomfort from the needle scratch.
- *Blood sample* – we would like to obtain a sample of up to 30ml (6 teaspoons) of blood for assessing markers of inflammation. By markers we mean substances that include molecules called proteins, lipids and messenger RNA. Messenger RNA can help us understand how genes function. These and other molecules can affect disease processes (in this case asthma) and how patients like you respond to medicines. In addition, we may need to use a small amount of the blood to look at a specific substance (IgE) that is increased if you are allergic to certain things.
- *Urine* - we would like to collect a sample of your urine. We will use this to check your exposure to cigarette smoke (i.e. whether you smoke, how much you smoke or if you are passively exposed to cigarette smoke).

Visit 2 – Southampton General Hospital (approximately 4 hours)

This visit to the Wellcome Trust Clinical Research Facility will be arranged at a time that is convenient for you. During this visit you will be asked to have a bronchoscopy.

What is a bronchoscopy?
A bronchoscopy is a routine diagnostic test which allows the doctor pass a small fibreoptic telescope through the nose to the back of the throat and down the trachea (windpipe) to examine the large airways of the lungs. A tiny pair of forceps can be passed into the lungs to take samples. These will be analyzed to help determine the cells and molecules involved in Asthma and Allergy. It is normally a very safe and well tolerated procedure.

How do I prepare?
You will need to attend the the Wellcome Trust Clinical Research Facility usually at 9am having fasted (not had anything to eat or drink) for at least 4 hours. You can take your inhalers as normal and any essential medications 2 hours before with a small sip of water. If you are unsure of what to do regarding any of your medications you should discuss this with the nurse or doctor at the initial visit or telephone us for advice. Please bring your inhalers and medications with you. If you are unwell in the week before the procedure is scheduled please contact a member of the research team to see if it should be rebooked.

What happens before and during the procedure?
Prior to the procedure we will insert a small plastic tube (cannula) into your hand or arm and use this to give you medication to make you feel drowsy (Midazolam) and reduce coughing (Alfentanyl). In addition to this we will spray your nose and the back of your throat with anaesthetic spray to numb them so that they are not irritated by the procedure. This preparation takes up to 15 minutes, the anaesthetic tastes unpleasant and may make you cough but this passes off quickly. We will be monitoring your heart rate, blood pressure and oxygen levels throughout this time. We then pass the bronchoscope down one of the nostrils, through the voicebox and into the main airways of the lung, giving more anaesthetic spray on the way. Some people have small nasal passages and in these circumstances we pass the bronchoscope through the mouth.
After inspecting the airways we would take some samples. There are 3 types.

1. Lavage/wash – a small amount of fluid will be flushed into a corner of the lung and then sucked out again. This will allow us to collect free cells within the airways. You may get a taste of salty water in your mouth.
2. Brushing – a small brush will be gently rubbed against the airway wall to remove some cells.
3. Biopsy – small forceps are used to sample the airways – there are no nerve endings here so this is not painful. Up to 10 biopsies may be taken, about 1-2mm in size, smaller than a pin-head.

This part of the procedure will take 20-30 minutes on average. With the sedating medications some people go off to sleep while others are pleasantly drowsy. If you wanted us to stop the procedure at any time we would do so.

What happens after the procedure?
After the procedure you will be kept under observation for 2-3 hours. During this time you will be checked regularly by our experienced bronchoscopy nurses. If necessary we may give you nebulised Salbutamol or a course of steroids. After 2 hours you will be allowed to have something small to eat and drink and then discharged home.
There is a very small chance that you might need to stay for longer or overnight. As you have been given a sedative you will need a responsible friend or family member (over 16) to accompany you home but if this is not possible one of our Isle of Wight research nurses could do so. You should not drive or operate heavy machinery for 24 hours after the procedure. We would also advise against drinking alcohol for 24 hours after the procedure.

What are the risks and discomforts?
It is very common to cough up small flecks of blood for the 48 hours after a bronchoscopy. This is nothing to worry about. If it persists longer than this you should contact a member of the research team.
Common risks

- Local discomfort – sore throat or sore nose during or after the procedure
- Fever/chills
- Dizziness after the sedative injection
- Mild chest pain (10 in 100)
- Temporary worsening of asthma requiring steroids (20 in 100)
- Pain or infection at IV site (1 in 100)

Uncommon risks (less than 1 in a 1000 procedures)

- Bleeding
- Spasm of major airways/vocal cords
- Infection (pneumonia/bronchitis)
- Irregular heartbeat

One death has been reported after a research bronchoscopy in the US, but not in the UK. Many thousands of research bronchoscopies have been performed, so the risk of death is extremely remote.

If clinically relevant information is obtained at the time of the bronchoscopy this will be shared with you and the relevant doctors involved with your care. The samples obtained will be anonymised and the results will not be put in your medical records.

This completes the information about the Main Study; if you are considering taking part please continue to read the additional information in Part 2 before making any decision.

PART 2

This section will answer some of your questions and tell you more about the conduct of the study and what you can expect during the study.

1. WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART IN THIS STUDY?

You would not receive any benefits directly however your participation in this study will allow us to see how asthma and allergies affect children and teenagers as they grow up. It may help us develop new treatments for these medical problems in the future.

2. WILL EXPENSES BE PAID?

Reasonable time and travel expenses you (and your escorting family member or friend) may have incurred as a result of participation in this study will be reimbursed up to a maximum of £300 The study team will discuss this with you.

3. WHAT ARE THE ALTERNATIVES FOR DIAGNOSIS AND OR TREATMENTS?

The purpose of this study is to further our understanding of asthma so that improved treatments and ways of diagnosing and monitoring asthma may be developed in the future.

4. WHAT ARE THE SIDE EFFECTS OF ANY TREATMENT RECEIVED WHEN TAKING PART?

This study does not include a new or experimental drug.

5. WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?

The possible disadvantages and risks of each of the tests which you may be asked to participate in have been detailed in this information sheet.

6. WHAT IF THERE IS A PROBLEM?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. Please raise your concerns in the first instance with the Principal Investigator (that is the lead researcher), Professor Hasan

Arshad. His contact details are at the end of this form. If you wish to make a more formal complaint, please contact the hospital's Patient Advice and Liaison Service (available 9 am to 4.30 pm Monday to Friday, out of hours there is an answer phone). Tel: 023 8079 8498, Email: PALS@suht.swest.nhs.uk

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against the Southampton University Hospital Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanism will still be available to you. As the Principal Investigator is an employee of the University of Southampton, additional professional indemnity and clinical investigation insurance is in place.

If you have any questions or concerns, please contact Professor Hasan Arshad or Sharon Matthews at The David Hide Asthma and Allergy Research Centre, St Mary's Hospital, Newport, Isle of Wight. Telephone: 01983 534897. Email: IOWstudy@iow.nhs.uk.

8. WILL TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information which is collected about you during the course of the study will be treated confidentially. All study data related to you will be coded. This means that you will be given a code number that will be used to identify you and information about you without having to use your name, medical record number, or other common identifiers. If you give your consent to participate in this study, you also give your consent for access to your medical file. However, any information that may identify you will never leave the David Hide Asthma and Allergy Centre.

We do need to let your GP know you are taking part in this study. If any clinically relevant information is obtained as a result of your involvement in the study we will discuss this with you. . With your permission we would send your GP the results of your allergy and lung challenge tests as they may be useful for your future medical care. We would not send your GP any other results from the study.

All samples that are provided by you will be uniquely identified with a sample identifier and your code. If you change your mind about participating in the research, the code and link to your identity will allow us to locate your samples and destroy them so they cannot be used for further research.

The Southampton University Hospital Trust who is monitoring the study may also wish to access records as part of their monitoring of ongoing research.

It is possible that the data and results will be audited by a government agency that oversees this work to check that we are performing the work correctly. In this case there may be a need to access your protected information.

9. WHAT WILL MY STUDY DATA BE USED FOR?

Your study data will be used for research into asthma and may be used in research related to the development of pharmaceutical products, diagnostics or medical aids. The handling of your study data will be in accordance with applicable Data Protection law(s).

10. WHAT WILL HAPPEN TO THE SAMPLES I GIVE?

Blood: we will use this to check how allergic you are and use it to look at proteins and other cells that could be related to the development and control of asthma. Additionally we will extract genetic material from your cells. Cells in your body contain a type of molecule called deoxyribonucleic acid, or DNA for short. DNA is what your genes are made of. Genes are inherited and direct growth, development, and how the body functions. For example, some genes control the colour of your hair or eyes. Scientists have learned a lot about how genes work. There are many differences, or variations, in DNA from one person to another. These variations may affect a person's chance of suffering from a particular disease. You are being asked to donate a blood sample for genetic research that will determine part of the structure of your DNA and enable us compare it with medical information about you. We are asking you and other subjects participating in the study to donate a blood sample because we want to study how genetic differences are involved in asthma and allergy. Some of your DNA may be stored to look at other genes identified in asthma and allergy in the future.
Urine: we plan to measure the level of cotinine in this sample, this increases if you have been exposed to cigarette smoke, passively or otherwise. The result will not be released to your parents or doctors.
Bronchoscopy samples (bronchioalveolar lavage, brushings and biopsies): we will use these samples to see how well the cells in your airways recover from being damaged. We will extract the genetic material from the sample and look for genes and gene changes in relation to asthma and allergy.

Samples will be stored securely at the David Hide Asthma and Allergy Centre until they are analysed. Only the researchers at the centre will have access to them. Some of these blood and bronchoscopy samples will be analysed at the Southampton General Hospital, by researchers who work for the University of Southampton. The samples will not be labelled with your name or address so that the researchers analysing them will not know that the sample belongs to you. With your permission, we would like to store some blood and samples for use in further studies into asthma and allergic disease. We will only use these stored samples for studies reviewed and approved by the Local Research Ethics Committee. The movement and storage of samples will be carried out in accordance with the Human Tissue Act 2004.

11. WHAT IF RELEVANT NEW INFORMATION BECOMES AVAILBLE?

Sometimes during the course of a research study, new information becomes available. On receiving new information your research doctor might consider it to be in your best interest to withdraw from the study. He/she will explain the reasons and arrange for

your care to continue. If the study is stopped for any reason, you will be told why and your continuing care will be arranged.

12. WHAT WILL HAPPEN IF I DO NOT WANT TO CARRY ON WITH THE STUDY?

You can decide to withdraw from the study at any time. Refusal to take part or withdrawal after giving your consent will have no consequences for your present or future treatment by your doctor. Information collected may still be used. If you ask, we will destroy any stored samples that can still be identified as yours.This will not impact on the treatment and care that you are or will be receiving in any way.

13. WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

We aim to publish the results of the study in medical journals so that other doctors and researchers can make use of them. This is likely to be accompanied by an article in the local press on the Isle of Wight. It will not be possible to identify any individual involved in the study from these published results.

.14. WILL THERE BE ANY FINANCIAL BENEFITS TO ME FROM TAKING PART IN THE STUDY AND FROM THE RESULTS AND SAMPLES USED IN THIS STUDY AND IN ANY FUTURE RESEARCH?

Any information derived from this research, any patents, diagnostic tests, drugs, or biological products developed as a result of this research, are the property of the study sponsors (and its successors, licensees, and assigns) and partners in the study. These may be used for commercial purposes. You have no right to benefit from this property or to any share of the profits that may be earned directly or indirectly from this research.

However, in signing this form you do not give up any other rights that you would otherwise have as a participant in research.

15. WHO IS ORGANISING AND FUNDING THE RESEARCH?

The researchers at The David Hide Asthma and Allergy Research Centre are organising and carrying out this study. The Medical Research Council is funding this research.

16. WHO HAS REVIEWED THE STUDY?

The Research Ethics Committee of IOW, Portsmouth & SE Hampshire has approved this study.

17. CONTACT DETAILS & SOURCE FOR ADDITIONAL INFORMATION.

If you have any questions or concerns, please contact Prof Hasan Arshad or Sharon Matthews at The David Hide Asthma and Allergy Research Centre, St Mary's Hospital, Newport, Isle of Wight. Telephone: 01983 534897. Email: IOWstudy@iow.nhs.uk.

APPENDIX 1

FEMALE PARTICIPANTS

All female participants will be asked to perform a urinary pregnancy test before the lung challenge test and (if more than a week has elapsed) before the bronchoscopy also. This is because the effects of these procedures on a pregnant woman and an unborn baby are not known. This simply involves providing us with a sample of your urine and the test takes 5 – 10 minutes to perform.

If you are planning to become pregnant during the time of the study we would recommend that you do not take part. Any woman who becomes pregnant during the course of the study should contact a member of the research team as soon as possible.

What are the implications of undergoing a pregnancy test?

Before you consent to having a pregnancy test, we would like you to take time to consider the implications of taking the test and the result you may receive. A urine pregnancy test is a screening test only and you may require further testing to confirm your pregnancy.

If you test positive and you were planning to be pregnant then this may be good news for you. However, if you were not expecting to test positive, we understand that you might have mixed feelings about being pregnant.

Unplanned pregnancies happen and every woman has the right to decide for herself how to deal with the situation. The result will not be shared with anyone e.g. parent, guardian or GP without your consent.

What support is available in the event of a positive pregnancy test?
Trained staff will be available at the Allergy Centre to discuss, in confidence, the implications or your pregnancy test and direct you to the appropriate sources of further confidential guidance and professional support available through the Sexual Health Service, St Mary's Hospital (Tel: 01983 534202) or your GP. Alternatively The British Pregnancy Advisory Service (BPAS) website www.bpas.org.uk offers valuable information.

	Patient Information sheet	
Southampton / NHS Isle of Wight	Study number	RHM MED0956 REC 10/H0501/66

Do I have to take part?
It is your decision whether or not you wish to take part. If you do, you will be asked to sign a consent form. A decision to withdraw at any time, or a decision not to take part, will not affect the medical care you receive.

What if there is a problem or require further information?
If you have any questions or concerns, please contact Prof. Hasan Arshad or Sharon Matthews at The David Hide Asthma and Allergy Research Centre, St Mary's Hospital, Newport, Isle of Wight PO30 5TG. Telephone: 01983 534897 or e-mail: IOWstudy@iow.nhs.uk.

Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential.

Southampton NHS University Hospitals NHS Trust | NHS Isle of Wight | University of Southampton School of Medicine

INFORMED CONSENT FORM

Title of study: MRC-EMTU Study 2 – IOW Bronchoscopy study
Name of Principal Investigator: Hasan Arshad
Participant ID:

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

PART A: Consent for the current study

PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I have read the information sheet version 4.0 dated 12th April 2012 for the above study and have been given a copy to keep. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐☐
3. I agree to give a sample of blood and urine for research in this study. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time. ☐☐
4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. I understand that the information will be kept confidential. ☐☐
5. I understand that my GP may be informed of my participation and also the results of lung challenge and allergy test. ☐☐
6. I understand that I will not benefit financially if this research leads to the development of a new treatment or test. ☐☐
7. [FEMALE PARTICIPANTS ONLY] I have read the participant information sheet Appendix 1 and all of my concerns regarding pregnancy test have been answered to my satisfaction. I am ready to proceed with pregnancy tests prior to the lung challenge test and Bronchoscopy. ☐☐
8. I know how to contact the research team if I need to. ☐☐
9. I agree to participate in this study ☐☐

Participant: name surname	Date	Signature
Researcher taking consent: name surname	Date	Signature

Original for Investigator Site File , 1 copy for participant, 1 copy for medical record/hospital notes

THE DAVID HIDE ASTHMA AND ALLERGY RESEARCH CENTRE

Initial: [ ][ ][ ] | **IoW-Bronchoscopy Study** | **STUDY NO** [ ][ ][ ][ ]

ISAAC QUESTIONNAIRE FOR ALL SUBJECTS

Date of birth [ ] Date seen [ ] Male [ ] Female [ ]

I would like to ask you some questions about your home and family

1. Is your house Owned privately [ ]
   Rented privately [ ]
   Rented-council/house assn [ ]
   Other [ ]

   Do you cook on Gas [ ]
   Electric [ ]
   Other [ ]

2. Are you still living with your parents / guardians? Yes [ ] No [ ]
3. Are you still in education? Yes [ ] No [ ]
4. Do you work? Yes [ ] No [ ]
   If yes, please specify: [ ]
5. Total annual family income (estimate)
   Less than 12,000 [ ]
   12,000 – 17,999 [ ]
   18,000 – 29,000 [ ]
   30,000 – 41,999 [ ]
   greater than 42,000 [ ]
6. Does your home have damp spots on the walls or ceiling? Yes [ ] No [ ] Don't know [ ]
7. Does your home have visible moulds or fungus on the walls or ceiling? Yes [ ] No [ ] Don't know [ ]
8. *SMOKING inside the house*
   Mother Yes [ ] No [ ] [ ] /day
   Father Yes [ ] No [ ] [ ] /day
   Other Yes [ ] No [ ] [ ] /day

Outside the house (eg garden or porch) Yes [ ] No [ ]
Other places Yes [ ] No [ ]

9. *PETS in last 2 2 years*

Cat	Yes including bedroom [ ]	Yes, not bedroom [ ]	Yes, not in house [ ]	No [ ]
Dog	Yes including bedroom [ ]	Yes, not bedroom [ ]	Yes, not in house [ ]	No [ ]
Other	Yes including bedroom [ ]	Yes, not bedroom [ ]	Yes, not in house [ ]	No [ ]

Others what? [ ]

Regular exposure elsewhere (more than once a week)
Cat Yes [ ] No [ ]
Dog Yes [ ] No [ ]
Other Yes [ ] No [ ] What? [ ]

10. How frequently are you annoyed by outdoor air pollution (from traffic, industry, etc.) in your home if you keep the windows open?
    Every day [ ] Once a week [ ] Once a month [ ] Once a year [ ] Never [ ]

Bronchial Challenge Test (Mannitol)

Study No:

Initials

Pre-Challenge

	1	2	3	4	5	Best Effort
FVC						
FEV1						

Baseline - 0mg Capsule

	1	2	Actual Drop (ml)	% Drop	Best Effort
FEV1			- N/A -	- N/A -	

Using best attempt calculate target FEV1 (15% drop)

FEV1 for each dose

	1	2	Actual Drop (ml) from Baseline	% Drop from Baseline	Actual Drop (ml) from Previous Dose	% Drop from Previous Dose	Best Effort
Dose 2 - 5mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 3 - 10mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 4 - 20mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 5 - 40mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 6 - 80mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 7 - 160mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 8 - 160mg			- N/A -	- N/A -	- N/A -	- N/A -	
Dose 9 - 160mg			- N/A -	- N/A -	- N/A -	- N/A -	

PC15 =

Southampton School of Medicine | Isle of Wight NHS Primary Care Trust | Southampton NHS University Hospitals NHS Trust

MRC-EMTU study 2

Study No: ☐☐☐☐☐
Initials: ☐☐

MRC-EMTU study 2 (epidermal mesenchymal trophic unit)

CASE REPORT FORM

Study number _____

Initials _____

Cohort group

Persistent asthma	
Asthma remission	
Late-onset asthma	
Never asthma	

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton School of Medicine | Isle of Wight NHS Primary Care Trust | Southampton NHS University Hospitals NHS Trust

MRC-EMTU study 2

Study No: ☐☐☐☐☐
Initials: ☐☐

Preparatory information

Patient letter (& PIS) sent ☐☐/☐☐/☐☐ (DD/MM/YY)

Patient reply received ☐☐/☐☐/☐☐ (DD/MM/YY)
Method: e-mail/text/telephone call/reply slip returned

Contact details and preferred time of contact

Screening telephone call ☐☐/☐☐/☐☐ (DD/MM/YY)

Screening questions

Are you interested in taking part in the study? Yes/No

Are you available to visit the IOW and Southampton for 2 separate visits? Yes/No

Do you currently have asthma? Yes/No

Is patient in correct cohort group? Yes/No
If no: changed to

Is patient still eligible for study? Yes/No

First visit arranged?
Yes ☐☐/☐☐/☐☐ (DD/MM/YY) No ☐

Patient verbally reminded about pre-visit preparation Yes ☐

(No caffeine for 4 hours, no respiratory infection within last 14 days, not taking steroids, no inhaled short-acting β-agonist for 8 hours, no inhaled corticosteroids/Atrovent for 12 hours, no LABA/combination inhalers/theophylline for 24 hours, no Spiriva/antihistamine for 72 hours, no Montelukast for 4 days)

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton School of Medicine | Isle of Wight NHS Primary Care Trust | Southampton NHS University Hospitals NHS Trust

MRC-EMTU study 2

Study No: ☐☐☐☐☐
Initials: ☐☐

RECRUITMENT DATE

Date ☐☐/☐☐/☐☐ (DD/MM/YY)

Study number: ☐☐☐☐

Cohort

Persistent asthma	
Asthma remission	
Late-onset asthma	
Never asthma	

Exclusion criteria

Unable to attend for requisite 2 visits ☐

Pregnancy ☐

Checklist

Informed consent obtained ☐

Copy of consent form and information sheet filed in notes ☐

Copy of consent form and information sheet given to participant ☐

Original consent form placed in study file ☐

Letter sent to GP ☐

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton School of Medicine | Isle of Wight NHS Primary Care Trust | Southampton NHS University Hospitals NHS Trust

MRC-EMTU study 2

Study No: ☐☐☐☐☐
Initials: ☐☐

First meeting – David Hide Research Facility

Date ☐☐/☐☐/☐☐ (DD/MM/YY)

Preliminary Checklist
No caffeine for 4 hours ☐
No respiratory infection within last 14 days ☐
Not taking steroids ☐
Not taken medications for times specified:
- Short-acting β-agonist - 8 hours ☐
- Inhaled corticosteroids/Atrovent - 12 hours ☐
- LABA/combination inhalers/theophylline - 24 hours ☐
- Spiriva/antihistamine ☐
- Montelukast ☐

Pregnancy test Positive/Negative/Not applicable

Past medical history			
Drug history			
Asthma history			
Age = yrs	Height = cm	Weight = kg	
Observations			
Pulse =	BP =	Sats =	

Questionnaires
ISAAC ☐
Environmental questionnaire ☐

Urine for cotinine ☐
Smoker Yes/No /day
Log details

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton | Isle of Wight NHS | Southampton NHS

MRC-EMTU study 2

Study No: ☐☐☐☐☐☐

Initials: ☐☐

Allergen	Wheal	Mean Diameter
Histamine [1]		
N Saline [2]		
House Dust Mite PT [3]		
Grass pollen [4]		
Tree pollen [5]		
Cat [6]		
Dog [7]		
Alternaria Alternata [8]		
Cladosporium Herbarum[9]		
Milk [10]		
Egg [11]		
Soya [12]		
Cod [13]		
Wheat [14]		
Peanut [15]		

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton | Isle of Wight NHS | Southampton NHS

MRC-EMTU study 2

Study No: ☐☐☐☐☐☐

Initials: ☐☐

Bronchial challenge test (Mannitol)

Test result Positive/negative P15 ☐☐☐

Record of medications given ☐☐☐☐☐☐☐☐☐☐

Post-test FEV1: ☐☐☐ % of pre-test FEV1: ☐☐☐
Must be >95%

Second visit booking (Southampton)

Notes re patient availability/away dates etc

Booking made for

Date ☐☐/☐☐/☐☐ (DD/MM/YY) Time ☐☐:☐☐

Patient given verbal instructions re preparation Yes☐

Fasting for 4 hours, essential medications can be taken, inhalers can be taken, check not diabetic, check not on Warfarin/aspirin, directions

Written information sent out to patient Yes☐

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton | Isle of Wight NHS | Southampton NHS

MRC-EMTU study 2

Study No: ☐☐☐☐☐☐

Initials: ☐☐

Second meeting – Wellcome Trust Southampton

Date ☐☐/☐☐/☐☐ (DD/MM/YY)

Pre-bronchoscopy checklist

Specific informed consent taken	Yes☐	No☐
FEV_1 >1.0L	Yes☐	No☐
Patient fasted for 4 hours	Yes☐	No☐
Saturations on room air >92%	Yes☐	No☐
Transport and escort arranged (and patient has someone to stay with overnight)	Yes☐	No☐
Is patient diabetic?	Yes☐	No☐
(if so have specific instructions been given re medications)	Yes☐	No☐
Is participant on Warfarin, Aspirin or other 'blood-thinners?'	Yes☐	No☐
(if so have necessary adjustments been made?	Yes☐	No☐

General observations

Pulse= RR= BP= Sats=

Examination ☐☐☐☐☐☐☐☐☐☐

Pregnancy test Positive/Negative/Not applicable

Blood taken Yes☐ No☐

Log details ☐☐☐☐☐☐☐☐☐☐

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

Southampton | Isle of Wight NHS | Southampton NHS

MRC-EMTU study 2

Study No: ☐☐☐☐☐☐

Initials: ☐☐

Bronchoscopy procedure

Procedure commenced		☐☐:☐☐
Sedation	Midazolam	☐mg
	Alfentanyl	☐mcg
Total lignocaine		☐mg
Bronchoalveolar lavage	Volume infused	☐ml
	Volume returned	☐ml
Brush biopsy		☐
Endobronchial biopsies (number)		☐
Procedure ended		☐☐:☐☐

Complications/post procedure medications

☐☐☐☐☐☐☐☐☐☐

Other notes/special instructions

☐☐☐☐☐☐☐☐☐☐

Serious Adverse Events (SAE) reported? Yes ☐ No☐
If answer yes, complete relevant form

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2. 1st August 2011. REC Number: 10/H0501/66

MRC-EMTU study 2

Study No: ☐☐☐☐☐☐
Initials: ☐☐

Telephone follow-up

Date ☐☐/☐☐/☐☐ (DD/MM/YY)

Time ☐☐:☐☐

Serious Adverse Events (SAE) reported? Yes ☐ No☐
If answer yes, complete relevant form

Outcome of study:

Completed EMTU ☐
Withdrawn from EMTU ☐
Reason

MRC-EMTU study 2

Study No: ☐☐☐☐☐☐
Initials: ☐☐

Additional sheets

Date ☐☐/☐☐/☐☐ (DD/MM/YY)

Time ☐☐:☐☐

Serious Adverse Events (SAE) reported? Yes ☐ No☐
If answer yes, complete relevant form

Additional details

MRC-EMTU Study 2 IOW Bronchoscopy
Version number 2: 1st August 2011. REC Number: 10/H0501/66

APPENDIX III Markers of Remodelling the Wessex Severe Asthma cohort: a pre-study project

Samples from 24 participants were selected for immunohistochemical analysis (see Methods chapter); 2 were rejected as the biopsy was insufficient for analysis leaving 22 samples. 10 were from asthmatic participants (4 with mild asthma, 1 with moderate asthma and 5 with persistent severe asthma) and 12 from healthy controls (figure 14). Each biopsy was sampled twice.

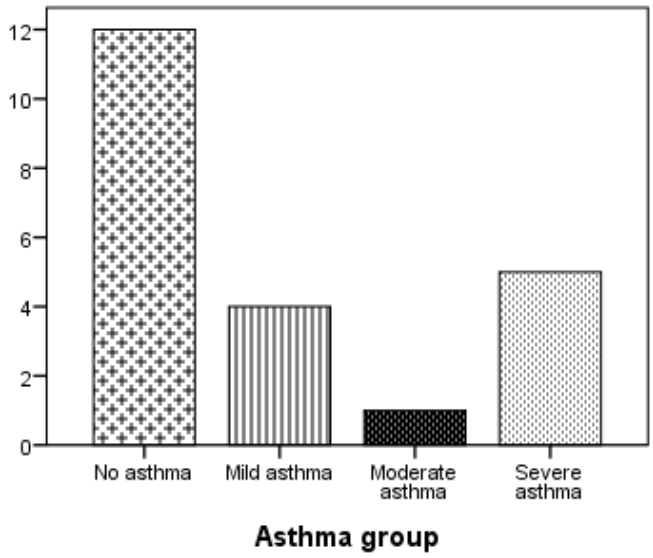


Figure 14: Asthma group of samples analysed in the Wessex Severe Asthma Cohort

COLLAGEN BAND THICKNESS

Collagen band thickness (CBT) was measured in 9 asthmatic subjects and 12 non-asthmatic subjects. The mean CBT in asthmatic subjects was 11.20µm and non-asthmatic subjects 9.30µm. Analysis by independent samples t-test revealed this difference to be significant ($p = 0.045$; 95% CI: 0.49 – 3.76) (figure 15).

Stratifying by asthma severity, the mean collagen band thickness in each asthma group and in the healthy control group has been summarised in table 25. The difference in CBT between non-asthmatic subjects and those with mild or moderate asthma was not statistically significant but the collagen band thickness in subjects with severe asthma was significantly more than non-asthmatic subjects ($p <0.001$) and those with mild asthma ($p = 0.017$) (figure 16).

Figure 15: Boxplot of collagen band thickness (µm) in each asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

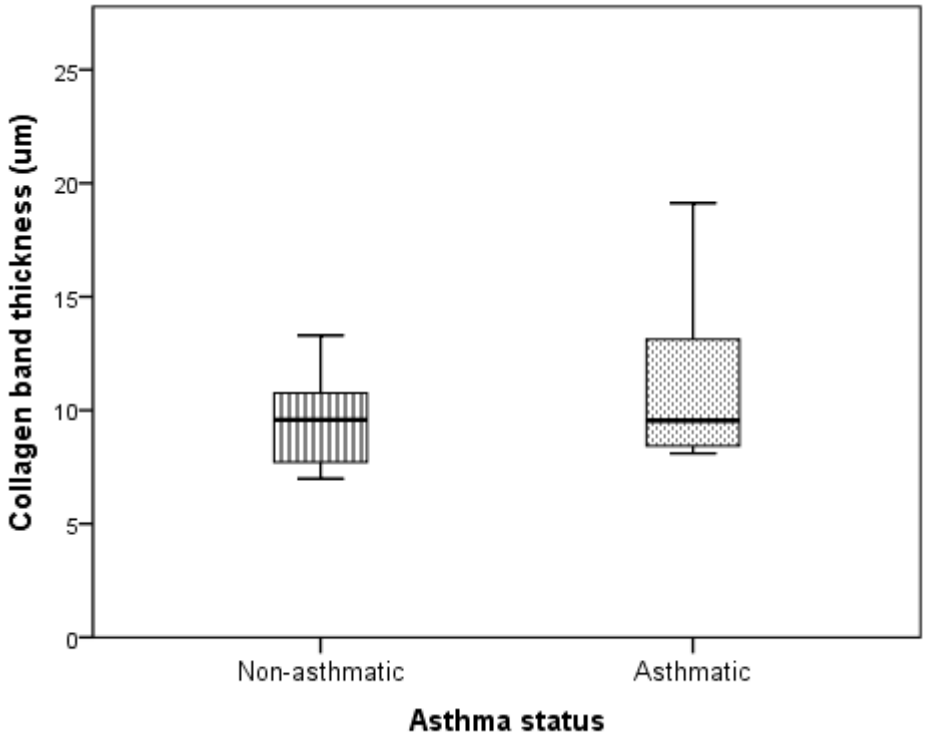


Figure 16: Boxplot of collagen band thickness (µm) between asthma severity groups compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

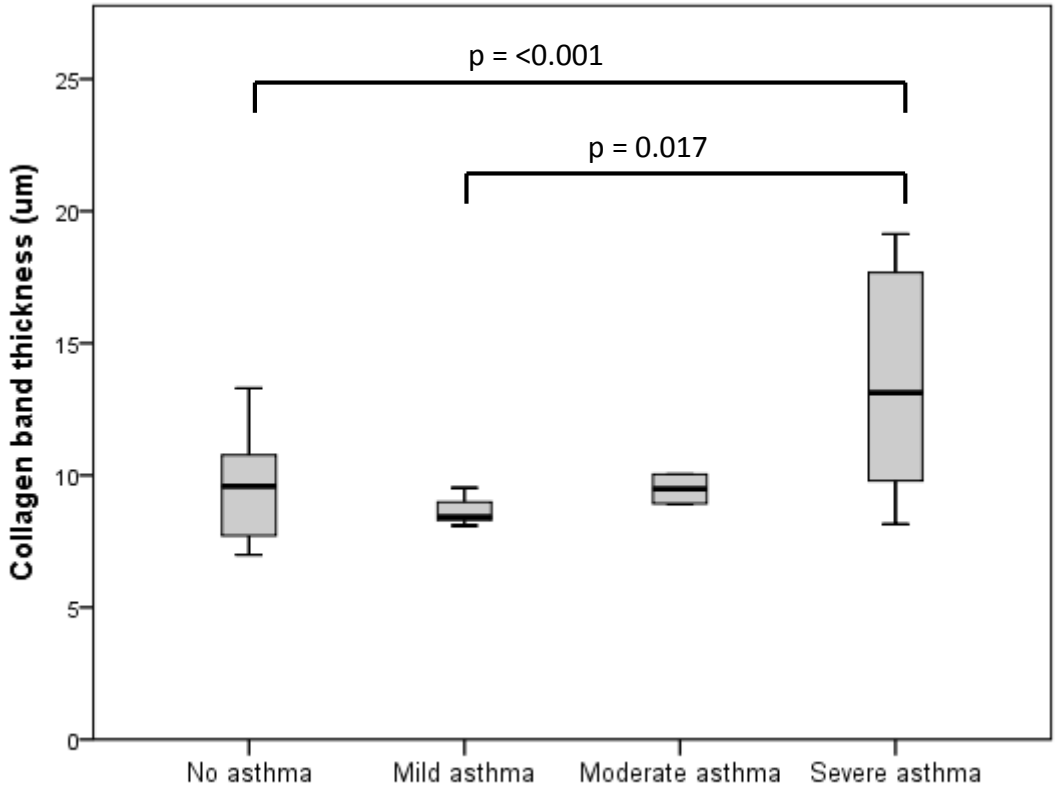


Table 26: Mean collagen band thickness (µm) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohort

Asthma group	N	Mean CBT (µm) and standard deviation
Healthy control	12	9.30 (SD: 1.80)
Mild asthma	3	8.63 (SD: 0.53)
Moderate asthma	1	9.49 (SD: 0.78)
Severe asthma	5	13.56 (SD: 4.30)

SUBEPITHELIAL COLLAGEN I DEPOSITION

Subepithelial collagen I was measured in all subjects. Mean collagen I deposition for those with asthma was 30.83% and for the healthy controls was 27.82% (figure 17). Analysis by independent samples t-test revealed this difference not to be significant (p = 0.154).

Stratifying by asthma severity, mean collagen I (%) in each asthma group and in the healthy control group has been summarised in table 26. Those with no asthma and mild asthma have a lower percentage of collagen I than those with moderate or severe asthma. The difference between those subjects with no asthma and those with severe asthma is statistically significant (p = 0.031) and the difference between those with mild asthma and those with severe asthma is approaching significance (0.077) (figure 18).

Figure 17: Boxplot of subepithelial collagen I deposition (%) in asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

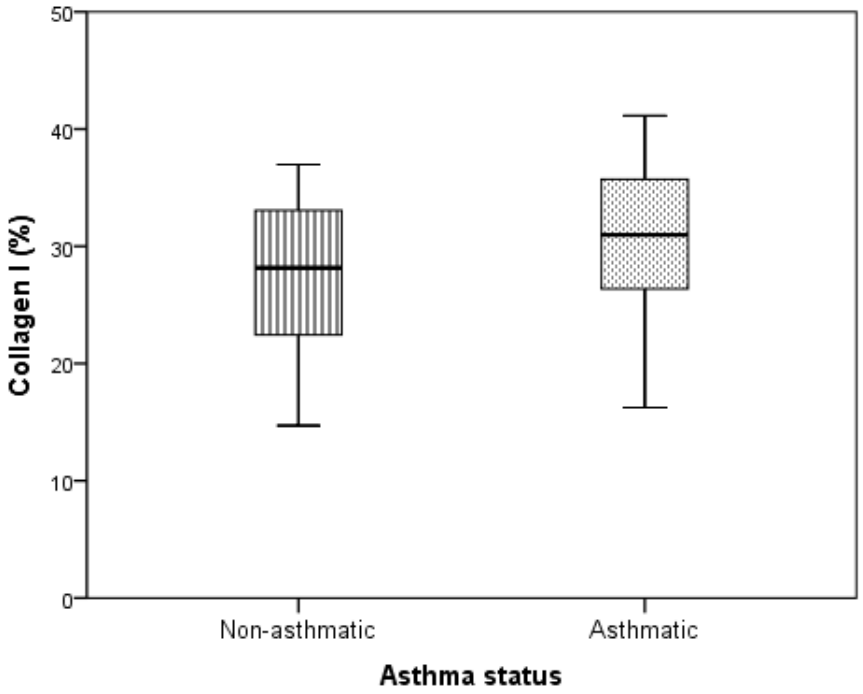


Figure 18: Subepithelial collagen I deposition (%) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohort

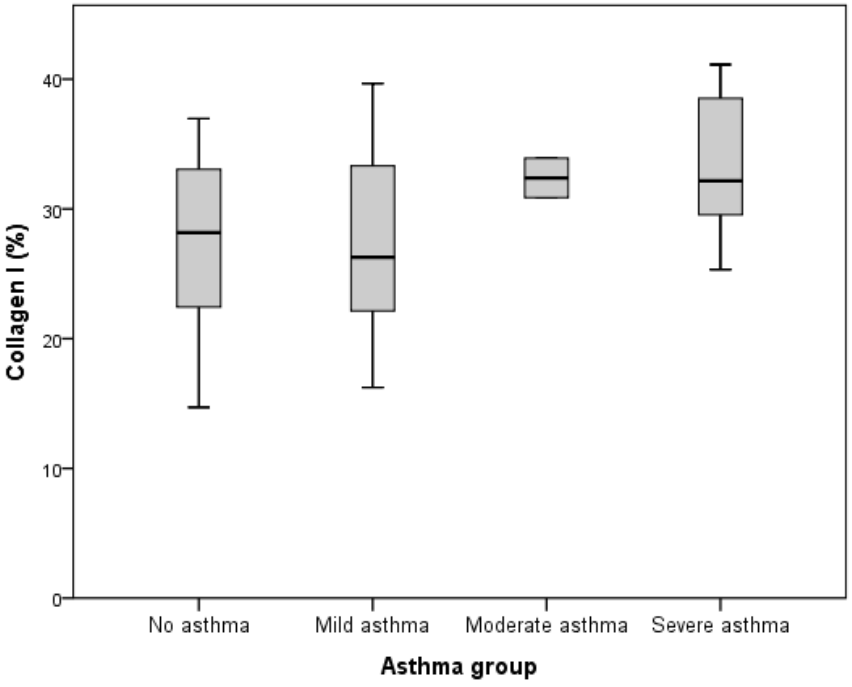


Table 27: Mean subepithelial collagen I deposition (%) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohort

Asthma group	N	Mean Collagen I % and standard deviation
Healthy control	12	27.82% (SD: 6.64)
Mild asthma	4	27.42% (SD: 7.85)
Moderate asthma	1	32.39% (SD: 2.14)
Severe asthma	5	33.25% (SD: 5.21)

SUBEPITHELIAL COLLAGEN III DEPOSITION

Subepithelial collagen III was measured in 7 asthmatic subjects and 12 non-asthmatic subjects. Mean collagen III deposition for those with asthma was 31.35%% and for the healthy controls was 29.09% (figure 24). This difference was not significant (p = 0.316).

Stratifying by asthma severity, mean collagen III (%) in each asthma group and in the healthy control group has been summarised in table 27. Although the percentage of collagen III was higher in those with severe asthma than in the other groups, the differences between the groups were not statistically significant (summarised in figure 25).

Figure 24: Boxplot of subepithelial collagen III deposition (%) in asthmatic compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

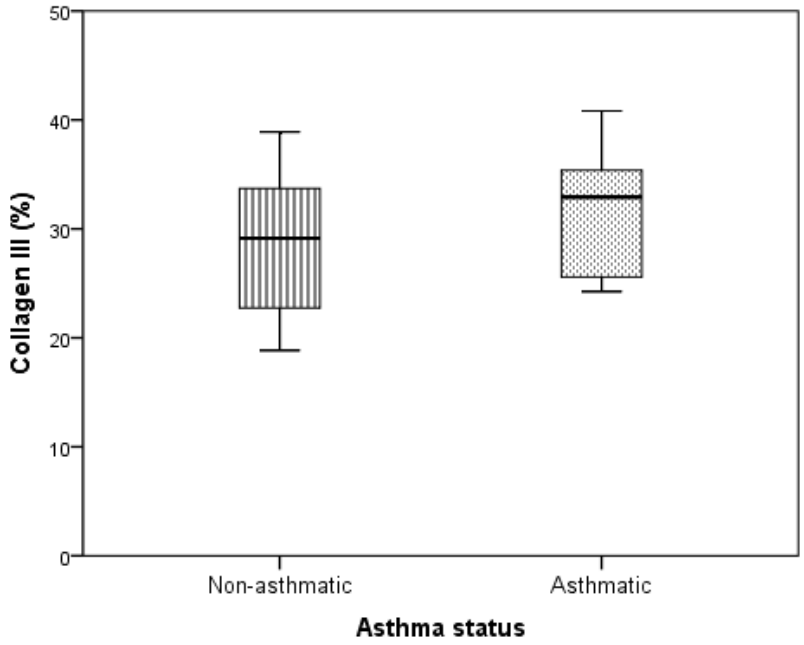


Figure 25: Boxplot of subepithelial collagen III deposition (%) between asthma severity groups compared to non-asthmatic subjects in the Wessex Severe Asthma Cohort

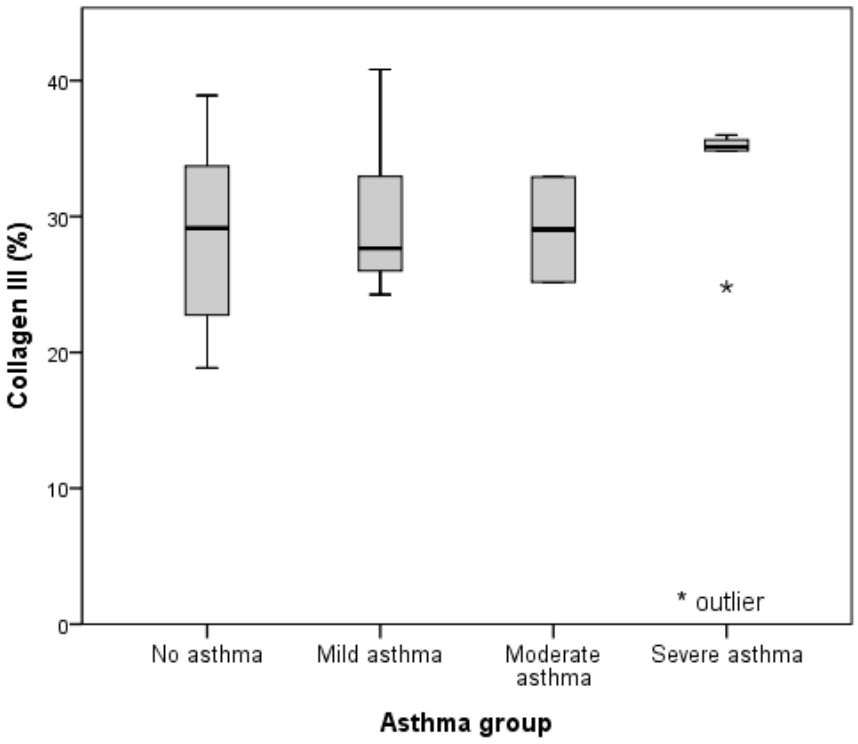


Table 28: Mean subepithelial collagen III deposition (%) in each asthma severity group and non-asthmatic subjects in the Wessex Severe Asthma Cohort

Asthma group	N	Mean Collagen III (%)and standard deviation
Healthy control	12	29.09% (SD: 6.17)
Mild asthma	3	30.33% (SD: 6.70)
Moderate asthma	1	29.04% (SD: 5.47)
Severe asthma	3	33.28% (SD: 4.75)

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