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UNIVERSITY OF SOUTHAMPTON
FACULTY OF MEDICINE
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Determinants of Airway Remodelling in Asthma

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ABSTRACT

FACULTY OF MEDICINE

SCHOOL OF MEDICINE

Doctor of Philosophy

DETERMINANTS OF AIRWAY REMODELLING IN ASTHMA

by Christopher Grainge

Asthma is a common disease characterised clinically by episodes of bronchoconstriction and pathologically by underlying structural airway changes called airway remodelling. Airway remodelling is associated with worse clinical outcomes and has been associated with eosinophilic inflammation. *In vitro* models have however suggested that mechanical stress associated with bronchoconstriction may induce airway remodelling in the absence of inflammation. This possibility has been investigated in human asthmatic volunteers.

48 asthmatics were randomised to one of 4 inhalation challenge protocols each involving 3 challenges at 48 hr intervals: allergen challenge (bronchoconstriction and eosinophilic airway recruitment), methacholine (bronchoconstriction without eosinophilic inflammation), saline (no bronchoconstriction) and, as an additional control to account for non-bronchoconstrictor effects of methacholine, salbutamol followed by methacholine (no bronchoconstriction). The airways were sampled with bronchoalveolar lavage and bronchial biopsy at fibre optic bronchoscopy prior to and 4 days after completing the last challenge.

The allergen challenges produced no adverse clinical consequences and produced similar immediate bronchoconstriction as the methacholine challenges. The 2 control challenges did not induce any bronchoconstriction. Inflammation increased only in the allergen group, with increases in bronchoalveolar lavage eosinophils ($p=0.01$), eosinophil cationic protein ($p=0.002$) and tissue eosinophils ($p=0.05$), whilst both allergen and methacholine groups showed significant induction of airway remodelling not seen in the 2 control groups. Sub-basement membrane collagen thickness ($p<0.001$) and epithelial mucus staining ($p=0.003$) as well as cell division in the epithelium ($p=0.001$) and the submucosa ($p<0.001$) all increased. There were no differences between the allergen and methacholine groups.

Additionally, using an *in vitro* model it was also demonstrated that asthmatic epithelium responds to mechanical stress by producing more transforming growth factor beta than normal epithelium ($p=0.04$).

Bronchoconstriction is sufficient to induce airway remodelling in asthma, and the asthmatic epithelium is abnormal in its response mechanical stress. These findings have important implications for the assessment, investigation and management of asthma.

Contents

ABSTRACT	I
LIST OF FIGURES	IX
LIST OF TABLES.....	XIII
DECLARATION OF AUTHORSHIP	XVII
ACKNOWLEDGEMENTS	XIX
LIST OF ABBREVIATIONS	XXI
CHAPTER 1 INTRODUCTION.....	1
1.1 Asthma - a historical perspective	1
1.2 Why study asthma	6
1.3 Pathology of asthma	7
1.4 Airway remodelling	8
1.5 Structure of the airway.....	8
1.5.1 The Epithelium	8
1.5.2 The basement membrane	9
1.5.3 The extracellular matrix.....	10
1.5.4 The airway smooth muscle.....	10
1.6 Role of acetylcholine in the airway.....	11
1.6.1 Vascular remodelling.....	14
1.7 Potential mediators of airway remodelling	15
1.7.1 TGF β	15
1.7.2 TGF β and airway remodelling	16
1.7.3 The Adipokines	18
1.7.4 Osteopontin.....	26
1.8 Clinical impact of airways remodelling	28
1.9 Airway remodelling - pathological, or maligned and misunderstood? ...	30
1.10 Causes of airway remodelling.....	32
1.11 Eosinophils in asthma and remodelling	34
1.11.1 Eosinophil depletion studies.....	36
1.11.2 Animal models of remodelling implicating the eosinophil	37
1.11.3 Non TGF β mediated eosinophil pathways in remodelling	38
1.12 Role of the epithelium	39
1.13 Airway response to mechanical stress.....	40
1.14 Epithelial response to compressive stress.....	42
1.15 Epithelial signal transduction of mechanical compression	44
1.15.1 Mechanochemical signal transduction;	44
1.16 Project rationale.....	47
CHAPTER 2 MATERIALS AND METHODS	49
2.1 Patient recruitment and selection.....	49

2.2	Skin prick testing.....	51
2.3	Methacholine challenge (screening).....	51
2.4	Allergen challenge.....	52
2.5	Methacholine challenge (repeated).....	53
2.6	Saline challenge.....	54
2.7	Salbutamol and methacholine challenges.....	54
2.8	Symptom scores.....	55
2.9	Bronchoscopy.....	55
2.9.1	Bronchial biopsies.....	55
2.9.2	Bronchial brushings.....	55
2.9.3	Bronchoalveolar lavage.....	56
2.9.4	BAL processing.....	56
2.9.5	BAL cell counts.....	56
2.10	Processing of tissue into glycol methacrylate.....	56
2.11	Sectioning of GMA embedded tissues.....	57
2.12	Staining procedure for GMA embedded tissue.....	57
2.13	Periodic acid-Schiff staining.....	63
2.14	Image analysis.....	63
2.14.1	Image presentation.....	64
2.15	ELISA.....	70
2.15.1	RELM β ELISA.....	71
2.16	Statistical analysis.....	72
2.17	RNA extraction.....	73
2.17.1	Homogenisation of bronchial biopsy tissue.....	73
2.17.2	Trizol extraction of cells grown on transwells.....	73
2.17.3	RNA extraction (adapted from TRIzol® Reagent protocol).....	74
2.18	DNase treatment.....	75
2.19	Quality check and quantitation of RNA.....	75
2.20	Reverse Transcription.....	76
2.20.1	Annealing step.....	76
2.20.2	Extension step.....	77
2.21	Quantitative real time polymerase chain reaction (RT qPCR).....	77
2.21.1	Theory of RT qPCR.....	77
2.22	Genes investigated by rtPCR.....	80
2.23	Analysis of RTqPCR data by $\Delta\Delta C_T$ method.....	84
2.24	Cell Culture - Air Liquid Interface cultures.....	85
2.24.1	Growing cells at an Air Liquid interface.....	85
2.24.2	ALI culture minimal medium.....	86
2.25	Cell compression system.....	86
2.25.1	System establishment.....	94
2.25.2	Initial experiments.....	94
2.25.3	System optimisation.....	94
2.26	Cell compression.....	95
CHAPTER 3	AIRWAY CHALLENGE STUDY.....	97
3.1	Background.....	97
3.2	Aims.....	100
3.3	Methods and Results.....	100
3.3.1	Characterisation of volunteers.....	100
3.3.2	Repeated airway challenges.....	108

3.3.3	Safety	108
3.3.4	Magnitude of the early asthmatic reaction.....	108
3.3.5	Magnitude of the late asthmatic reaction.....	115
3.3.6	Recovery of FEV ₁ prior to repeated challenge	122
3.3.7	Dose of provocant required to cause FEV ₁ drop	124
3.3.8	Symptom score	126
3.3.9	Reliever use	129
3.4	Discussion	132
3.4.1	Repeated methacholine challenges	135
3.4.2	Repeated saline challenges	135
3.4.3	Repeated salbutamol / methacholine challenges	135
3.4.4	FEV ₁ prior to repeated challenge	136
3.4.5	Symptom scores and Reliever use.....	136
3.4.6	Further work.....	137

CHAPTER 4 INFLAMMATION AS A RESULT OF REPEATED INHALATION CHALLENGES 139

4.1	Background	139
4.1.1	Interleukin 8.....	141
4.1.2	Eosinophil cationic protein.....	142
4.2	Aims.....	144
4.3	Methods and results	144
4.3.1	Volunteers.....	144
4.3.2	Repeated bronchoscopy	144
4.3.3	Challenge spirometry	150
4.3.4	Bronchoalveolar lavage.....	155
4.3.5	BAL ELISA measurements of inflammation	164
4.3.6	Immunohistochemistry	168
4.3.7	Epithelial cells	170
4.3.8	Submucosal cells	172
4.4	Discussion	179
4.4.1	Repeated bronchoscopy	179
4.4.2	Completed bronchoscopy groups.....	181
4.4.3	BAL responses to repeated inhalation challenge	181
4.4.4	Tissue responses to repeated inhalation challenge	182
4.5	Summary	187

CHAPTER 5 REMODELLING RESPONSES TO REPEATED INHALATION CHALLENGES 189

5.1	Background	189
5.1.1	Eosinophils and mechanical stress as potential initiators of airway remodelling	190
5.1.2	Epithelial activation in asthma	191
5.1.3	Ki-67.....	192
5.1.4	Measurement of airway remodelling	192
5.2	Aims.....	193
5.3	Methods and Results.....	193
5.3.1	Volunteers.....	193
5.3.2	Samples	194
5.3.3	Immunohistochemistry	194

5.3.4	Remodelling markers	208
5.3.5	Bronchoalveolar lavage (BAL) ELISA measurements of markers of remodelling	223
5.3.6	Serum measurements	233
5.3.7	Reverse transcriptase PCR results	234
5.3.8	Resistin like molecule beta (RELM β) in health and disease	245
5.4	Discussion	249
5.4.1	Epithelial activation following allergen and methacholine challenges	249
5.4.2	Threshold for response to bronchoconstriction?	251
5.4.3	Role of eosinophils in airway remodelling	252
5.4.4	Airway permeability changes following challenges	253
5.4.5	Why no qPCR results?	255
5.4.6	Limitations of the current study	257
5.4.7	A new asthma paradigm?	259
5.4.8	Remodelling, a normal response to mechanical stress?	264
CHAPTER 6	IN VITRO MODELLING OF BRONCHOCONSTRICTION	267
6.1	Background	267
6.2	Aims	272
6.3	Methods and results	272
6.3.1	<i>In vitro</i> model of bronchoconstriction	272
6.3.2	Establishment of the model system	272
6.3.3	Volunteer recruitment	273
6.3.4	Bronchoscopy and cell culture	273
6.3.5	Experimental design	273
6.3.6	Results	274
6.4	Discussion	282
6.4.1	Absent response from normal cells to compression	282
6.4.2	Further work	285
CHAPTER 7	CONCLUSIONS	287
7.1	Implications for early treatment of asthma and lung development	287
7.2	The role of bronchodilators in airway remodelling	289
7.3	The EMTU and airway remodelling	290
7.4	Measurement of airway hyperreactivity in asthma	292
7.5	Should these findings change our management of clinical asthma patients?	293
7.6	Resistin like molecule beta (RELM β)	294
7.7	Limitations of this study	295
7.8	Future work	297
7.9	Summary	299
CHAPTER 8	APPENDICES	301
8.1	Study exclusion criteria	301
8.2	Symptom diary card	303
8.3	Main study volunteer characteristics	305
8.4	Characteristics of volunteers in RELM β study (Immunohistochemistry and BAL)	310

8.5	Characteristics of volunteers in RELM β study (qPCR analysis).....	315
8.6	Characteristics of volunteers used in <i>in vitro</i> study.....	318
8.7	Cell culture media	321
8.8	Published work	324
CHAPTER 9 REFERENCES		333

List of figures

Figure 1-1 Putative mechanisms of activation of MAPKinase and PI3-Kinase by muscarinic M2 and M3 receptors in airway smooth muscle.....	13
Figure 1-3 Ribbon diagrams of resistin and resistin like molecule beta (RELM β).	21
Figure 1-4 Schematic diagram of the buckling effect of airway smooth muscle constriction on the airway epithelium and basement membrane.	30
Figure 1-5 Schematic diagram of coculture model of epithelial constriction leading to fibroblast response.....	44
Figure 2-1 RELM β immunostaining at low power with recombinant protein preabsorption control.	61
Figure 2-2 RELM β immunostaining at high power with isotype antibody control.	62
Figure 2-3 Method of calculating length of epithelium in bronchial biopsy.	65
Figure 2-4 Method of calculating biopsy area.	66
Figure 2-5 Method of calculating submucosal collagen area.	67
Figure 2-6 Method of calculating percentage of positive periodic acid Schiff staining.	68
Figure 2-7 Method of delineating collagen band thickness.	69
Figure 2-8 Result from nanodrop ND 1000 spectrophotometer.	76
Figure 2-10 Amplification and melt curves of Sybr Green RT qPCR	79
Figure 2-11 Schematic diagram of cell pressurisation system.....	88
Figure 2-12 Custom manufactured compression apparatus.	89
Figure 2-13 Home made and manufactured humidification apparatus.....	90
Figure 2-14 Pressure manifold and bungs.	90
Figure 2-15 Method for sealing and pressurising transwells.....	91
Figure 2-17 Multiple bungs inserted into transwell plate for simultaneous pressurisation of multiple transwells.	91
Figure 2-18 The cells are contained inside a acrylic enclosure which is filled with 5% CO ₂ in air.	92
Figure 2-19 The pressurisation apparatus inside a temperature and humidity controlled microscope housing.	93
Figure 3-1 Study timeline	101
Figure 3-2 Schematic diagram of study design.....	101
Figure 3-3 Age of volunteers by exposure group.	104
Figure 3-4 Body Mass Index (BMI) of volunteers by exposure group.	104
Figure 3-5 Forced expiratory volume in 1 second of volunteers as percentage of predicted value by exposure group.....	105
Figure 3-6 Percentage predicted forced vital capacity of volunteers by exposure group.	105
Figure 3-7 Skin prick test wheal diameter in response to histamine in volunteers by exposure group.	106
Figure 3-8 Skin prick test wheal diameter in response to house dust mite in volunteers by exposure group.	106

Figure 3-9 Provoking concentration of methacholine required to induce a 20% fall in forced expiratory volume in 1 second in volunteers by exposure group.	107
Figure 3-10 Percentage change in FEV ₁ over time following first of three allergen, methacholine, saline and salbutamol / methacholine challenges.	117
Figure 3-11 Percentage change in FEV ₁ over time following second of three allergen, methacholine, saline and salbutamol / methacholine challenges.	117
Figure 3-12 Percentage change in FEV ₁ over time following third of three allergen, methacholine, saline and salbutamol / methacholine challenges.	118
Figure 3-13 Percentage change in FEV ₁ following repeated allergen challenge.	119
Figure 3-14 Percentage change in FEV ₁ following repeated methacholine challenge.	119
Figure 3-15 Percentage change in FEV ₁ following repeated saline challenge..	120
Figure 3-16 Percentage change in FEV ₁ following repeated salbutamol and methacholine challenge.	120
Figure 3-17 Mean change in FEV ₁ following three repeated allergen, methacholine, saline and salbutamol / methacholine challenges each 48 hours apart.	121
Figure 3-18 Percentage change in FEV ₁ prior to repeated allergen, methacholine, saline or salbutamol / methacholine challenges.	123
Figure 3-19 Inhaled doses of allergen or methacholine required to induce a 15% fall in FEV ₁ on three consecutive inhalation challenges each separated by 48 hours.....	125
Figure 3-20 Mean daily symptom scores in weeks prior to and during repeated allergen, methacholine, saline or salbutamol / methacholine challenges.	127
Figure 3-21 Mean daily reliever use in weeks prior to and during repeated allergen, methacholine, saline or salbutamol / methacholine challenges.	131
Figure 4-1 Eosinophils as a percentage of total cells recovered at bronchoalveolar lavage following repeated inhaled challenges.	161
Figure 4-3 Eosinophil cationic protein (ECP) measured in bronchoalveolar lavage fluid following repeated inhaled challenges.....	166
Figure 4-4 Immunohistochemical staining of eosinophils with anti EG2 antibody.	168
Figure 4-5 Immunohistochemical staining of macrophages using an anti CD68 antibody.....	169
Figure 4-6 Immunohistochemical staining of mast cells using an anti AA1 antibody.....	169
Figure 4-7 Eosinophils per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges.	174
Figure 4-8 Mast cells per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges.....	177

Figure 5-1 Immunohistochemical staining of TGF β using a anti pan TGF β antibody.	195
Figure 5-2 Immunoexpression of transforming growth factor beta (TGF β) expressed as percentage of total epithelial area following repeated inhaled challenges.	197
Figure 5-3 Immunohistochemical staining of resistin like molecule beta (RELM β) using an anti RELM β antibody.	199
Figure 5-4 Immunoexpression of resistin like molecule beta (RELM β) expressed as percentage of total epithelial area following repeated inhaled challenges.	201
Figure 5-5 Immunohistochemical staining of cells with anti Ki-67 antibody (arrow).	203
Figure 5-6 Ki-67 positive cells per millimetre of bronchial epithelium following repeated inhaled challenges.	205
Figure 5-7 Immunohistochemical staining of cells with anti MMP9 antibody.	206
Figure 5-8 Immunohistochemical staining of collagen III.	209
Figure 5-9 Subepithelial basement membrane thickness in bronchial biopsies (μ m) before and after repeated inhaled challenges.	211
Figure 5-10 Histochemical staining of mucins using periodic acid-Schiff reagent.	213
Figure 5-11 Percentage of epithelium staining positive with periodic acid - Schiff (PAS) reagent following repeated inhaled challenges.	215
Figure 5-12 Ki-67 positive cells per square millimetre of bronchial submucosa before and after repeated inhaled challenges.	218
Figure 5-14 Immunohistochemical staining of tenascin.	222
Figure 5-15 Relative expression (calculated by $\Delta\Delta$ CT method) of multiple genes of interest before and after repeated inhaled allergen challenge.	236
Figure 5-16 Relative expression (calculated by $\Delta\Delta$ CT method) of multiple genes of interest before and after repeated inhaled methacholine challenge.	237
Figure 5-17 Relative expression (calculated by $\Delta\Delta$ CT method) of multiple genes of interest before and after repeated inhaled saline challenge.	238
Figure 5-18 Relative expression (calculated by $\Delta\Delta$ CT method) of multiple genes of interest before and after repeated inhaled salbutamol then methacholine challenge.	239
Figure 5-19 Percentage total epithelium immunoexpressing resistin like molecule beta (RELM β) in bronchial biopsies from healthy volunteers and subjects with mild and severe asthma.	247
Figure 6-1 Schematic diagram of coculture model of epithelial constriction leading to fibroblast response.	270
Figure 6-2 <i>In vitro</i> model of bronchoconstriction developed by Choe and colleagues.	270
Figure 6-3 Total TGF β 2 concentration in basolateral medium of human bronchial epithelial cells from normal and asthmatic volunteers 24 hours after sham or real compression for 1 hour at 30cm water pressure.	276
Figure 6-4 Change in total TGF β 2 concentration in basolateral medium of human bronchial epithelial cells from normal and asthmatic volunteers 24 hours after sham or real compression for 1 hour at 30cm water pressure.	278

Figure 6-5 Mucociliary differentiation of primary human bronchial epithelial cells during ALI culture.	284
Figure 7-1 The growth pattern of expected FEV ₁ /FVC ratio in boys and girls with and without airways hyperreactivity.....	288

List of tables

Table 1-1 Naming conventions for the resistin family of proteins.....	21
Table 2-1 Primary antibodies used in immunohistochemistry, their source, chromogen and working dilution.	59
Table 2-2 Commerical ELISA kits used for sample analysis.....	70
Table 2-3 Amplification protocol for real time PCR using SYBR Green detection chemistry. Adapted from Primer Design recommendations.....	81
Table 2-5 Amplification protocol for real time PCR using Perfect Probe detection chemistry. Adapted from Primer Design recommendations.....	81
Table 2-7 Primer sequences for real-time PCR analysis.	82
Table 2-9 Amplification protocols.....	83
Table 3-1 Volunteer baseline characteristics	103
Table 3-2 Percentage falls in FEV ₁ following allergen, methacholine, saline or salbutamol / methacholine challenges.	110
Table 3-3 Comparison of change in FEV ₁ during early and late asthmatic reaction following repeated inhalation of allergen or methacholine.....	111
Table 3-4 Area under the curve analysis of FEV ₁ following allergen, methacholine, saline or salbutamol / methacholine challenges.	112
Table 3-5 Comparison of area under the curve for change in FEV ₁ during early and late asthmatic reaction following repeated inhalation of allergen or methacholine.	113
Table 3-6 Change in percentage predicted FEV ₁ at three stages of salbutamol and methacholine challenge; prior to challenge, following salbutamol and following methacholine.	114
Table 3-7 Resting mean percentage predicted FEV ₁ prior to repeated allergen, methacholine, saline and salbutamol / methacholine challenges.....	123
Table 3-8 Change in dose of provocant required to induce 15% drop in FEV ₁ on each challenge day.....	125
Table 3-9 Mean daily symptom score in week before and week of repeated inhaled challenges.	127
Table 3-10 Mean change in symptom score and daily reliever use between week prior to and week of repeated allergen, methacholine, saline, and salbutamol / methacholine challenges.	128
Table 3-11 Mean daily reliever use in week before and week of repeated inhaled challenges.	130
Table 4-1 Subject characteristics of volunteers undergoing repeated inhaled allergen challenges who were able and unable to tolerate repeated bronchoscopy.	146
Table 4-2 Mean maximal falls in FEV ₁ during early and late asthmatic responses in volunteers undergoing repeated inhaled allergen challenge who were able and unable to tolerate repeated bronchoscopy.....	147
Table 4-3 Mean area under the curve for changes in FEV ₁ during early and late asthmatic responses in volunteers undergoing repeated inhaled allergen challenge who were able and unable to tolerate repeated bronchoscopy.	148

Table 4-4 Percentage predicted FEV ₁ prior to repeated inhaled allergen challenge in volunteers able and unable to tolerate repeated bronchoscopy.	148
Table 4-5 Mean daily symptom scores in volunteers undergoing repeated allergen challenge who were able and unable to tolerate repeated bronchoscopy.	149
Table 4-6 Mean daily reliever use in volunteers undergoing repeated allergen challenge who were able and unable to tolerate repeated bronchoscopy.	149
Table 4-7 Baseline volunteer characteristics of all subjects tolerating repeated bronchoscopy.	151
Table 4-8 Mean maximal changes in FEV ₁ following repeated inhalation challenges in subjects who completed repeated bronchoscopy.	152
Table 4-9 Area under the curve for changes in FEV ₁ following repeated inhalation challenges in subjects who completed repeated bronchoscopy.	153
Table 4-10 Mean maximal changes in FEV ₁ following repeated inhalation challenges with allergen and methacholine in subjects who completed repeated bronchoscopy.	154
Table 4-11 Area under the curve for changes in FEV ₁ following repeated inhalation challenges with allergen and methacholine in subjects who completed repeated bronchoscopy.	154
Table 4-12 Percentage of bronchoalveolar lavage fluid recovered at bronchoscopy before and after repeated inhaled challenges.	157
Table 4-13 Total cells recovered in bronchoalveolar lavage before and after repeated inhaled challenges.	158
Table 4-14 Total cells recovered at bronchoscopy per 100ml of recovered BAL fluid before and after repeated inhaled challenges.	159
Table 4-15 Percentage of eosinophils in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.	160
Table 4-16 Percentage of neutrophils in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.	162
Table 4-17 Percentage of macrophages in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.	162
Table 4-19 Percentage of lymphocytes in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.	163
Table 4-20 Percentage of epithelial cells as percentage of total cells recovered in bronchoalveolar lavage before and after repeated airway challenges.	163
Table 4-22 Concentration of eosinophil cationic protein in BAL fluid before and after repeated inhaled challenges.	165
Table 4-23 Concentration of Interleukin 8 in BAL fluid before and after repeated inhaled challenges.	167
Table 4-24 Eosinophils per millimetre of bronchial mucosa epithelium before and after repeated inhaled challenges.	170
Table 4-25 Macrophages per millimetre of bronchial mucosa epithelium before and after repeated inhaled challenges.	171
Table 4-26 Mast cells per millimetre of bronchial mucosa epithelium before and after repeated inhaled challenges.	171
Table 4-27 Eosinophils per square millimetre of bronchial submucosal tissue before and after repeated inhaled challenges.	173

Table 4-28 Mast cells per square millimetre of bronchial submucosal tissue before and after repeated inhaled challenges.....	176
Table 4-29 Macrophages per square millimetre of bronchial submucosal tissue before and after repeated inhaled challenges.....	178
Table 5-1 Epithelial immunoexpression of TGF β expressed as percentage of total epithelial area before and after repeated inhaled challenges.....	196
Table 5-2 Epithelial immunoexpression of RELM β expressed as percentage of total epithelial area before and after repeated inhaled challenges.....	200
Table 5-4 Ki-67 positive cells per mm length of epithelium before and after repeated inhaled challenges.....	204
Table 5-5 MMP9 positive cells per mm length of epithelium before and after repeated inhaled challenges.....	207
Table 5-6 Subepithelial basement membrane thickness in bronchial biopsies before and after repeated inhaled challenges.....	210
Table 5-7 Positive periodic acid - Schiff (PAS) staining in bronchial biopsies as percentage of total epithelial area before and after repeated inhaled challenge.	214
Table 5-9 Number of Ki-67 positive cells per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges.....	217
Table 5-11 Number of MMP9 positive cells per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges.	220
Table 5-12 Percentage of submucosal tissue staining positive for collagen III before and after repeated inhaled challenges.....	221
Table 5-13 α 2 macroglobulin concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	224
Table 5-14 Osteopontin (OPN) concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	227
Table 5-15 sTNFaR2 concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	228
Table 5-16 GRO α concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	229
Table 5-17 Activin A concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	230
Table 5-18 Dipeptidyl peptidase 1 (DPP1) concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	231
Table 5-19 Surfactant protein D (SPD) concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	232
Table 5-20 Galectin 3 concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.....	232
Table 5-21 Osteopontin concentrations in serum fluid before and after repeated inhaled challenges.....	233
Table 5-22 Collagen 3 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	235
Table 5-23 Collagen 1 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	240
Table 5-25 A disintegrin and metalloprotease 33 (ADAM33) relative gene expression in bronchial biopsies before and after repeated inhaled challenges.	241

Table 5-27 Alpha smooth muscle actin (ASMA) relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	241
Table 5-28 MUC5AC relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	242
Table 5-29 FOXA2 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	242
Table 5-30 Tenascin relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	243
Table 5-31 SPP1 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	243
Table 5-32 TGF β relative gene expression in bronchial biopsies before and after repeated inhaled challenges.....	244
Table 5-34 Differences in immunoexpression of epithelial RELM β and collagenous basement membrane thickness in bronchial biopsies from healthy volunteers and subjects with mild and severe asthma.	246
Table 5-29 Relative expression of RELM β mRNA in bronchial epithelial brushings from healthy volunteers and subjects with mild and severe asthma.....	248
Table 6-1 Total TGF β (pg/ml) in basolateral medium of human bronchial epithelial cells from asthmatics and normal volunteers 24 hours after 1 hour of sham or real compression at 30cm water pressure.	275
Table 6-2 Difference in total TGF β (pg/ml) in basolateral medium between cells compressed at 30 cm of water pressure for 1 hour, and those subject to sham compression.	277
Table 6-3 Active TGF β (pg/ml) in basolateral medium of human bronchial epithelial cells from asthmatics and normal volunteers 24 hours after 1 hour of sham or real compression at 30cm water pressure.	279
Table 6-4 Relative TGF β 2 gene expression in human bronchial epithelial cells from asthmatics and normal volunteers after sham or real 1 hour compression at 30cm water pressure.....	280
Table 6-6 Relative RELM β gene expression in human bronchial epithelial cells from asthmatics and normal volunteers after sham or real 1 hour compression at 30cm water pressure.....	281

Declaration of authorship

I, Christopher Grainge declare that the thesis entitled Determinants of Airway Remodelling in Asthma and the work presented in it are my own work.

I confirm that;

- This work was done wholly or mainly while in candidature for a research degree at this university;
- Where any part of this thesis has been previously submitted for a degree or any other qualification at this university or other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always attributed;
- Where I have quoted the works of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- 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;
- Parts of this thesis have been published as:

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Grainge C, Howarth PH. Repeated high dose allergen challenge in asthma. CRJ 2010. First published online 7 JUN 2010 DOI: 10.1111/j.1752-699X.2010.00212.x

Copies of the published work are reproduced in the appendices.

Signed

Date

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List of abbreviations

A2	apolipoprotein A2
ACh	acetylcholine
AChR	acetylcholine receptor
ADAM	A disintegrin and metalloproteinase
AEC	3 - amino, 9 - ethylcarbazole
AHR	airway hyper responsiveness
ALI	air liquid interface
AQLQ	asthma quality of life questionnaire
ASM	airway smooth muscle
ASMA	alpha smooth muscle actin
AUC	area under the curve
BAL	bronchoalveolar lavage
BEBM	bronchial epithelial cell basal medium
BEGM	bronchial epithelial growth medium
BHR	bronchial hyperreactivity
BPE	bovine pituitary extract
BSA	bovine serum albumin
BTS	British Thoracic Society
CD	cluster of differentiation
cDNA	complimentary DNA
cm H ₂ O	cm of water pressure
COL1A1	collagen 1
COL3A1	collagen 3
C _t	threshold cycle
CTGF	connective tissue growth factor
CXCL	chemokine (C-X-C motif) ligand
DAB	diaminobenzidine
DC	dendritic cells
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate (mix of dATP, dCTP, dGTP and dTTP)
DPP1	dipeptidyl peptidase 1
EAR	early asthmatic reaction
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	endothelin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Egr-1	early growth response-1
ELISA	enzyme linked immunosorbent assay
EMTU	epithelial mesenchyme trophic unit
eNO	exhaled nitric oxide
EPO	eosinophil peroxidase
ET	endothelin
FAM	6-carboxy fluorescein

FBS	fetal bovine serum
FEV ₁	forced expiratory volume in 1 second
FGF	fibroblast growth factor
FIZZ	found in inflammatory zone
Fox p3	forkhead box p3
FRC	functional residual capacity
FVC	forced vital capacity
g	relative centrifugal force in gravities
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GINA	Global Initiative for Asthma
GMA	glycol methacrylate
GMCSF	granulocyte monocyte colony stimulating factor
GSDMB	gasdermin B
HBEC	human bronchial epithelial cell
HBEGF	heparin binding epidermal growth factor
HBSS	Hanks' buffered salt solution
HDM	house dust mite
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IGF	insulin like growth factor
IL	interleukin
IP-10	interferon gamma-induced protein 10kDa
IPF	idiopathic pulmonary fibrosis
IQR	inter quartile range
kd	kilodalton
LABA	long acting beta agonist
LAR	late asthmatic reaction
LT	leukotriene
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MMLV	moloney murine leukemia virus
MMP	matrix metalloproteinase
MUC5AC	mucin 5AC
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NO	nitric oxide
OPN	osteopontin
OVA	ovalbumin
PAS	Periodic acid-Schiff
PBS	phosphate buffered saline
PC ₂₀	provoking concentration required to induce 20% drop in FEV ₁
PDE4D	phosphodiesterase E3 dunce homolog Drosophila
PDGF	platelet derived growth factor
PEEP	positive end expiratory pressure
PG	prostaglandin
PI3K	phosphatidylinositol 3-kinase
PLL	poly-L-lysine
PPAR	peroxisome proliferator activated protein
qPCR	quantitative real time polymerase chain reaction
RBM	reticular basement membrane

RELM	Resistin like molecule
RNA	ribonucleic acid
ROW	reverse osmosis water
RT	reverse transcriptase
rtPCR	reverse transcriptase polymerase chain reaction
SABA	short acting beta agonist
SARA	smad anchor for receptor activation
SCF	stem cell factor
SMAD	SMA / Mothers against decapentaplegic homolog
SMART	Salmeterol multicentre asthma research trial
SPP1	osteopontin
STAT6	signal transducer and activator of transcription 6
sTNFaR2	soluble tumour necrosis factor alpha receptor 2
TAMRA	6-carboxy-N,N,N',N'-tetramethylrhodamine
TBS	tris buffered saline
TER	transepithelial resistance
TGF	transforming growth factor
Th1	T helper type 1 lymphocyte
Th2	T helper type 2 lymphocyte
TIMP	tissue inhibitor of metalloproteinase
TLC	total lung capacity
TLR	toll-like receptor
TMB	3-3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
TNXB	tenascin
TSLP	thymic stromal lymphopoietin
UBC	ubiquitin C
VEGF	vascular endothelial growth factor

Chapter 1 Introduction

1.1 Asthma - a historical perspective

Asthma, or a disease similar to it, has been described since antiquity. As early as 1550 BC the authors of the Ebers Papyrus recorded a concoction of herbs (frankincense, sesame and herbal apple) to be burned on a hot brick and the fumes inhaled to alleviate symptoms (Ghalioungui 1987, Marketos 1982).

Around a thousand years later, Hippocrates of Kos (c. 460 - 377 BC) described asthma in terms of a constellation of symptoms due to excess phlegm (one of the four humours) flowing into the lungs from the brain, and used a word first found in Homers Iliad to describe it; the Greek word for panting or gasping, 'asthmaino' (αστημαινω). Although some descriptions of conditions in the Hippocratic corpus probably relate to specific clinical entities (such as pleurisy) it is unclear whether 'asthma' referred to a disease, or more likely a constellation of symptoms. Hippocratic writers did note that the prevalence of asthma symptoms increased in the summer and autumn, though did not suggest a cause.

The Hippocratic tradition continued and was consolidated over the next several hundred years, especially with the work of Galen (129 - 216 AD) that further described the essential functions of the humours of phlegm, blood, yellow bile and black bile; this approach dominated medical thinking until the late Middle Ages. Despite being a master physician, Galen did not provide a lucid clinical picture of asthma, though he did separate asthma and dyspnoea in some circumstances, and described the spasmodic and also chronic nature of the breathlessness associated with asthma. Galen attributed asthma to a blockage of the trachea and bronchi by thick secretions due to an abnormal condition of the humours (Marketos 1982).

Even in the 1st Century AD there was not uniform agreement on how to address disease and its symptoms; Aretaeus of Cappadocia (81 - 131 AD) described asthma as an autonomous clinical entity (rather than a constellation of symptoms) characterised by paroxysms of breathlessness triggered by exercise and having cough as a major element. He noted that women were more susceptible to asthma, and put this down to them being 'humid and cold'. This approach to the disease did not fit into the accepted 'humours' paradigm; his work was ignored, and only rediscovered in 1550, at the beginning of the Renaissance.

Although little was published in science and medicine in Europe during the Middle Ages, Moses Maimonides (1137 - 1193 AD), a 12th century Rabbi, physician and philosopher working in Egypt, wrote extensively on the subject of asthma for his patron and patient, Prince Al-Afdal. He noted in his 'Treatise on asthma' that the Princes symptoms often began with a 'common cold' especially in the winter months and described how the patient was short of breath and had to gasp for air. Maimonides recommended the consumption of chicken soup as a remedy and avoidance of polluted city air as a preventative measure (Rosner 1987);

'city air is stagnant, turbid and thick, the natural result of its big buildings, narrow streets, (and) the refuse of its inhabitants..... the concern for clean air is the foremost rule in preserving the health of one's body'
(Rosner 1996)

With the Renaissance in Western European science, the Galenic and Hippocratic traditions were challenged by many, with the Swiss philosopher Phillip von Hohenheim (who changed his name several times, finally settling on the single word Paracelsus) (1493-1541) at the forefront of change. He combined a sober experimental approach with a volcanic temperament, and was apparently so averse to the Galenic tradition that he burnt the works of

Galen; his own writings promoted a change in the concepts underlying disease and its causes, provoking a more rational approach to the study of medicine (Siddiqui 2003). Fundamental to his thinking was the concept of fermentation as a central chemical reaction; The Belgian, Jean Baptiste van Helmont (1579 - 1644) applied Paracelsus' principles of fermentation, leading to his discovery of exhaled carbon dioxide. Baptiste was probably the first to suggest that the disease of asthma originated in the airways, and also argued against the accumulation of phlegm in the lungs causing asthma, as the onset of breathlessness was too rapid to be caused by its build up (Steel 2001).

English medicine was somewhat behind the advances on the continent early in the Renaissance, but Sir John Floyer (1649 - 1734), who suffered from asthma, published the first major English monograph on the subject in 1698, 'A Treatise of the Asthma.' He was writing as the Galenic tradition was being supplanted by more scientific explanation of disease processes in Britain – describing;

'the constriction of the bronchia, 'tis properly the periodic asthma, and if the constriction is great, it is with wheezing; but if less, the wheezing is not so evident.'

Despite this elegant description of bronchoconstriction, he still regards the underlying causes as essentially Galenic with;

'a flatulent slimy Caccochymia which is bred in the stomach creates an inflation there.... and in the membranes of the lung'

Floyer defined asthma clearly, separating it from other pulmonary disorders; in addition he considered dyspnoea due to bronchial constriction, with nocturnal symptoms and the presence of airflow obstruction, hyperinflation and wheeze. He also described the multifactorial basis of the asthma phenotype, identifying that asthma could be a seasonal disorder; but did not correlate this with the

presence of airborne antigen (Sakula 1984). It was not until 1831 that John Elliotson, Professor of Medicine at London University, proposed that pollen might be the environmental factor responsible. Though the seasonal nature of the disease waited for an explanation until the 19th century, the role of environmental factors in the triggering of asthma had been recognised in Britain nearly 300 years earlier, in 1547, by Geromo Cardano when he advised the asthmatic Archbishop of St Andrews to avoid feather bedding (Steel 2001).

Though there was an appreciation that environment could play a role in the aetiology of asthma, this understanding was first integrated into a whole disease paradigm by Henry Hyde Salter (1823 - 1871). He considered asthma a disease of the nervous system triggered by nervous action; this was due to previous work demonstrating that stimulation of the vagus nerve could induce bronchoconstriction. He did not consider mucosal oedema or mucus production to be significant as they were not present in post mortem studies of asthmatics that had died of other causes (Salter 1882, Diamant 2007, Steel 2001).

He described asthma in the 1860's as a;

'paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks' (with) 'hyperresponsiveness to cold air and exercise and attacks provoked by exposure to chemical and mechanical irritants, to particular kinds of air as well as to certain foods and wine'

William Osler (1849 - 1919) described airway hyperresponsiveness linked to exposure to non specific stimuli and also considered asthma an inflammatory disease on the basis of the presence of oedema, mucus and 'asthma crystals' in the sputum. These asthma crystals were later identified as eosinophilic granulocytes when Paul Ehrlich (1854 - 1915) discovered eosin (tetrabromofluorescein) (Diamant 2007).

In 1946 J. Curry established that asthmatics suffered reduced vital capacity following intravenous injection of histamine, and in the following year showed that intramuscular acetyl-beta-methyl choline had a similar effect. In a few patients in the later trial, Curry also administered the methyl choline by nebuliser, and demonstrated a more marked reaction than when administered parenterally. These studies gave the foundations to the universal tests for asthma, the histamine and methacholine challenges used today (Curry 1946 and 1947).

As with methacholine and histamine, the first deliberate administration of allergen to volunteers was not by the nebulised route, in this case however it was by administration into the skin. This was shown in some patients to cause respiratory symptoms, and led H. Herxheimer in 1951 to expose volunteers to inhaled allergen. In these experiments, Herxheimer, a surgeon working at University College London, administered cat dander, 'pollen of various kinds' and house dust mite extract by inhalation. The early and late asthmatic reactions were described in 74 patients, though rather than considering the possibility of an early and a late reaction in the same patient, the results were interpreted that the patients had either early or late reactions. Unlike Curry, vital capacity was not measured routinely, and reactions were characterised on the basis of symptoms. Patients who developed early reactions were treated with nebulised isoprenaline to relieve their symptoms, and any late reaction that developed was interpreted as occurring solely due to the isoprenaline wearing off. If a patient had no early symptoms, but did have late (defined as after approximately 4 to 6 hours) they were classified as being late reactors (Herxheimer 1952).

By the end of the twentieth century, the concept of asthma as a primarily inflammatory disorder was firmly established, with the presence of remodelling changes in the asthmatic airway noted, and thought to be due to secondary to ongoing inflammation. Since then, a paradigm shift has been proposed where

the chronic inflammatory processes in asthma are considered to occur related to, but in parallel with airway remodelling changes (Davies 2002).

1.2 Why study asthma

Asthma is a common chronic disease characterised by variable respiratory distress with underlying airway inflammation and airflow obstruction. Between 25% and nearly 30% of children in the United Kingdom have 'ever had asthma' by questionnaire studies (Devenney 2004, Burr 2006) and nearly 25% are routinely carrying inhalers (Burr 2006). In adults the prevalence of asthma is lower, but still increasing from 1.5% in the late 1970's to 6.9% in 2001-2004 (Browatzki 2009).

The incidence of asthma and allergic disease in the United Kingdom has risen inexorably over the last 50 years, in common with most of the developed world. Recent UK work has shown a quadrupling in numbers of children with peanut allergy between 1989 and 2001; over a similar period of time, hospital admissions due to anaphylaxis rose sevenfold (Gupta 2007). This short period of time does not allow for significant genetic drift, and therefore the rise in the incidence of these diseases is likely to be due to environmental changes. It is well known that asthma is worsened by exposure to environmental triggers which are commonly encountered in modern daily life, including air pollution, allergens and cold air (Johnston 2006).

Rapid changes in airway calibre can arise in asthma in response to irritant stimuli. Such narrowing is a reflection of the underlying hyperresponsiveness of the airway smooth muscle.

These responses can be mimicked by the inhalation of methacholine, which acts directly on the airway smooth muscle to induce airway narrowing and is not thought to involve inflammatory cell activation. Airway smooth muscle

contraction also arises as a consequence of exercise and/or inhalation of cold dry air, these stimuli promote mast cell degranulation and the subsequent airway smooth muscle narrowing is in response to the released mediators. This process can be mimicked by the inhalation of adenosine or mannitol. Mast cell degranulation is also an important feature of the airway response to allergen exposure. The situation with this stimulus is however, more complex, as the airway response also involves lymphocyte activation, eosinophil airway recruitment and epithelial cell activation. Epithelial activation by itself is a feature of exposure to low levels of air pollutants, such as ozone. It is thus apparent that different stimuli can preferentially influence different pathways that may lead to airway narrowing in asthma.

Whilst the influence of these triggers have been studied to evaluate their effects on airway inflammation, there is very little information as to how these differing exposures influence the airway structural changes that are considered relevant to the progression to disease chronicity.

1.3 Pathology of asthma

Asthma is a complex immunological disorder characterised by a specific form of inflammation of the airways mucosa, involving activated inflammatory cells (mast cells, eosinophils and T lymphocytes) and structural cells (epithelial cells, fibroblasts, smooth muscle cells and endothelial cells) whose function is altered in a process usually referred to as airway remodelling. Whilst a vast amount of knowledge has been accumulated over the last 20 years, the greatest advances have been made since the late 1980's with the introduction of fiberoptic bronchoscopy as a research tool to sample the airway mucosa (by bronchial biopsy) and bronchial lumen (by bronchoalveolar lavage).

1.4 Airway remodelling

Although it has been recognised for nearly 100 years that the airways of patients with asthma are abnormally thickened, the term airway remodelling has only become common since the late 1980's (McParland 2004). Airway remodelling is the process by which the structural, cellular and molecular components of the airway wall are altered in asthma. The process appears early in the disease, may predate clinical symptoms and involves all constituents of the airway wall including epithelial, submucosal and smooth muscle layers, as well as components not commonly considered structural, such as blood vessels (Pohunek 2005, Tang 2006).

1.5 Structure of the airway

The human airway differs in its cellular composition between the central and peripheral conducting airways, although the basic structure of the airway wall is maintained throughout. In cross section the airway consists of five distinct layers, those of epithelium, basement membrane, the subepithelial layer, the submucosa and the adventitia (Baroody 2003).

1.5.1 The Epithelium

In the respiratory tract the epithelium from trachea to small bronchi is pseudostratified and columnar. The most prevalent cells are ciliated epithelial cells that possess approximately 200 cilia extending into the liquid present on the epithelial surface. Goblet cells comprise a varying percentage of the epithelial cells, being more prevalent in the larger airways compared to the smaller. Clara cells are also present in the airways where they secrete surfactant, though these cells are only found peripherally and are usually absent from bronchoscopically obtained samples (Baroody 2003). Dendritic cells are also present in the epithelial surface of the airway, and extend

projections into the lumen in order to sample environmental antigens (Swindle 2009).

Both human and animal studies have demonstrated that the epithelium in asthmatics is both hyperplastic and metaplastic showing exaggerated differentiation into goblet cells (Fahy 2001). These changes are seen early in, or even prior to clinical onset of the disease (Sagiani 2005, Pohunek 2005). Though epithelial desquamation is also seen in asthmatics, it is not clear if this is an artefactual finding, and even if it is not, some studies have not shown any significant difference between asthmatics and controls (Carroll 1993, Lozewicz 1990). It is possible that the epithelium to basement membrane structures offer a tissue plane at which the epithelium detaches during tissue processing, however there remains the possibility that asthmatic epithelium has a more pronounced tissue weakness at this point, though this has yet to be investigated specifically. The new technique of atomic force microscopy, used in a force spectroscopy mode, would be well suited to the task (Muller 2008). As well as the structural changes of increased numbers and change in phenotype of cells, the epithelium is also functionally different in asthma, being more permeable to substance transfer including plasma proteins, more sensitive to oxidant stress and respiratory virus infection than in normal individuals (Bucchieri 2002, Bayram 2002, Knight 2002, reviewed in Holgate 2007). The epithelium is also a source for a wide variety of growth factors and cytokines which may influence other constituents of the airway (Davies 2002).

1.5.2 The basement membrane

The basement membrane is an extracellular layer found deep to the epithelium that is acellular, consisting of proteins including collagen types I, II, V and VII, as well as proteoglycans including fibronectin and laminin, which separates the airway epithelium from the mesenchyme. The basement membrane may be divided into three layers; the lamina lucida, the basal lamina and the lamina

reticularis (also known as the reticular basement membrane (RBM). Thickening of the lamina reticularis is a characteristic of asthmatic airway remodelling and involves deposition of collagen as well as glycoproteins (Roche 1989, Kariyawasam 2007). The deposition of these molecules in the RBM is probably due to the action of sub basement membrane myofibroblasts which may originate from the bone marrow (Benayoun 2003, Schmidt 2003). In normal individuals the subepithelial collagen layer is approximately 5 - 8 μm in depth, whereas in asthmatics it is approximately double this (McParland 2003). The thickening of the reticular basement membrane is positively correlated with airway smooth muscle (ASM) and mucus gland remodelling (James 2002).

1.5.3 The extracellular matrix

Tissue between and within the structures of the airway wall constitutes the extracellular matrix (ECM). The ECM functions to maintain structural integrity of the airway wall, to enable cellular migration, to secrete growth factors and cytokines, and to itself produce structural proteins and glycoproteins. The ECM in asthma shows increased deposition of such structural molecules, similar to those found in the RBM (James 2007). Increased mass of the ECM in asthma may contribute to the increase in volume of the airway smooth muscle that has also been reported (Oliver 2006).

1.5.4 The airway smooth muscle

The airway smooth muscle (ASM) causes dynamic airway narrowing in asthma, but airway smooth muscle cells are not purely contractile; they also determine the composition of the ECM and may participate in responses to allergen, infection and inflammation (Oliver 2006). ASM in asthma is hyperresponsive to normal stimuli (for example cold air, allergen), but the mechanism underlying this is unknown. Results of various studies examining ASM from asthmatics have shown no clear results suggestive that the ASM is hyper or hypo contractile, though some work has suggested that there is increase in myosin

light chain kinase in asthmatic ASM cells (Ma 2002). The mass of ASM is increased in asthma, and this appears to be as a result of both hypertrophy and hyperplasia, though up to 50% of the increase in the muscle mass may be due to an increase in the ECM between the ASM cells (Oliver 2006, Ebina 1993, McParland 2003). Airway smooth muscle cells may migrate and proliferate as well as secrete cytokines, extracellular matrix proteins and growth factors. The airway smooth muscle may also alter its phenotype from a normal contractile phenotype to a proliferative or synthetic phenotype and hence may play a role in the regulation of airway structure and airway remodelling (Gosens 2004).

1.6 Role of acetylcholine in the airway

The primary source for acetylcholine (ACh) within the airway is the vagus nerve; release of acetylcholine from its termini results in airway smooth muscle (ASM) constriction. The anticholinergic drugs such as ipratropium and tiotropium both block this action, so inducing bronchodilation. ACh acts at muscarinic acetylcholine receptors, which are G protein coupled receptors (unlike the ion channel linked nicotinic ACh receptors) classified according to their sensitivity to specific agonists and antagonists. At present, five receptor subtypes have been identified (M1-M5). The odd numbered receptors act via Gq (a heterodimeric G protein subunit that activates phospholipase C) which results in activation of the phosphatidylinositol trisphosphate pathway, and intracellular calcium mobilisation, and in addition, activation of protein kinase C. The even numbered receptors are coupled to Gi (also a heterotrimeric G protein subunit) and inhibit adenylyl cyclase activity. This receptor subtype also activates G protein gated potassium channels, leading to hyperpolarisation of the plasma membrane in different excitable cells (reviewed in Ishii 2006).

The release of acetylcholine from vagal nerve termini is regulated by a variety of prejunctional receptors, including autoinhibitory M2 receptors. It has been shown in animal models that there may be M2 autoreceptor dysfunction which

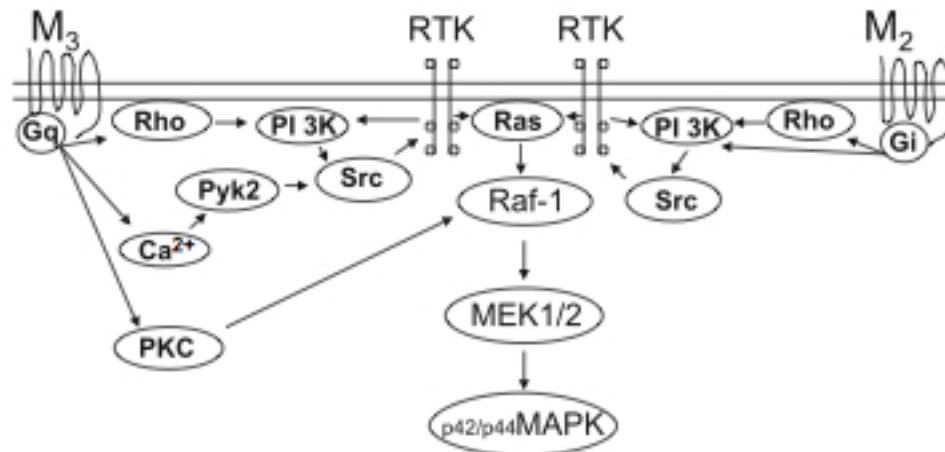
results in increased response to ACh release from the vagal nerve, both *in vitro* and *in vivo* (Ten Berge 1995). This M2 receptor dysfunction may be mediated by major basic protein (MBP) released from eosinophils (Adamko 1999) and these mechanism may be relevant in humans as M2 receptor dysfunction has been reported in some patients with asthma (Okayama 1994). Inflammatory mediators known to be present in the airways in asthma, including the prostaglandins D2 (PGD2) and F2 (PGF2) and thromboxane A2 have been shown to increase ACh release from cholinergic nerves by acting prejunctionally, and hence may enhance bronchoconstriction in asthma (Undem 2001).

The acetylcholine signalling system is also present on non neuronal tissues within the airway, with ACh being released from, degraded by cholinesterase and detected by both muscarinic and nicotinic ACh receptors on epithelial cells, T and B lymphocytes, mast cells, monocytes and alveolar macrophages. ACh may therefore act as a paracrine hormone within the airway (Wessler 2003, reviewed in Kawashima 2003).

Platelet derived growth factor (PDGF), epidermal growth factor (EGF) insulin like growth factor 1 (IGF-1) and fibroblast growth factor (FGF) are all implicated in airway remodelling, and rely on activation of MAP kinases and PI3Kinase for their effects. It has been shown that these pathways can be additionally activated by muscarinic agonists as shown at Figure 1-1.

Muscarinic stimulation alone appears insufficient to induce an increase in cell proliferation (as measured by ³H thymidine uptake) in human ASM cells, but muscarinic agonists have been shown to interact with peptide growth factors such as EGF causing synergistic induction of mitogenesis in ASM cells (Krymskaya 2000). This finding would suggest that acetylcholine release either from the vagus nerve, or from other sources in the airway might contribute, in the correct proinflammatory milieu, to airway remodelling.

Figure 1-1 Putative mechanisms of activation of MAPKinase and PI3-Kinase by muscarinic M2 and M3 receptors in airway smooth muscle.
From Gosens *et al* Eur J Pharm, 2004.



This possibility has been examined in animal models, and has shown that remodelling of the ASM (ASM mass, contractility and protein expression) after ovalbumin (OVA) challenge were decreased by the AChR antagonist tiotropium (Gosens 2005). It is notable that the tiotropium only altered ASM mass in animals exposed to OVA; the authors imply that this is due to the ACh only inducing remodelling where allergen induced inflammation is present. The possibility that bronchoconstriction alone could be inducing remodelling, and the tiotropium could be reducing bronchoconstriction and hence remodelling was not addressed. In further work with a similar model involving 12 weekly challenges with ovalbumin in sensitised guinea pigs, the increase in mucus gland hypertrophy was also diminished with tiotropium, as was the increase in tissue eosinophilia, there was however no increase in extracellular matrix deposition in this model (Bos 2007). These results suggest that remodelling of both the ASM and mucin glands may be driven in part by ACh release following

allergen challenge, though the reduction in bronchoconstriction could have led to a decrease in remodelling if bronchoconstriction alone is a stimulator of airway remodelling; an increase in mucin without inflammation but in the presence of mechanical stress mimicking bronchoconstriction has been demonstrated (Park 2009). *In vitro* work has shown that proliferation of fibroblasts is driven by muscarinic agonists, and that the response is driven by the p-42/44 MAPK pathway. Though the original work incubated both fibroblasts cell lines and primary human fibroblasts with carbachol for 24 hours in order to elicit a response, later work demonstrated that the response to muscarinic agonists is rapid (within 2 minutes) and the effect is blocked by both tiotropium and atropine, suggesting that the response is M2 and Gi mediated (Matthiesen 2006 and 2007). It has also been shown that immortalised human ASM cell lines (which express high levels of M2 and M3 receptors) respond to muscarinic agonists by the production of interleukin 8 (IL8) and IL6, with a synergistic effect with cigarette smoke extract to the secretion of IL8 (Gosens 2009).

Other cells within the airway also respond to cholinergic stimulation; when bovine primary bronchial epithelial cells are stimulated with methacholine and substance P independently, the epithelial cells secrete leukotriene B4 (LTB4) as well as other, unidentified eosinophil chemottractant factors (Koyama 1998)

1.6.1 Vascular remodelling

The number of blood vessels is increased in the airway wall in asthma, in addition to this, the thickness of the vessel walls increase as the permeability of the vessels also increases (Haczku 1997, Tigani 2007).

1.7 Potential mediators of airway remodelling

There are several potential mediators of airway remodelling including transforming growth factor beta (TGF β), resistin like molecule beta (RELM β) and osteopontin.

1.7.1 TGF β

The TGF β family of cytokines is classified into two subfamilies; the TGF β / activin / Nodal subfamily and the bone morphogenic protein / growth and differentiation factor / muellerian inhibiting substance subfamily. All members are related by sequence, and also by the signalling pathways that they activate (Shi 2003).

TGF β exists in a biologically active and an inactive form; the active form is a dimer composed of two TGF β monomers linked by a disulphide bond. Each TGF β monomer consists of several strands linked by three disulphide bonds, forming a structure known as the cysteine knot (Sun 1995).

TGF β binds to a serine/threonine kinase receptor complex consisting of two subunits, TGF β RI and TGF β RII. These receptor complexes themselves appear to function in pairs; a single TGF β molecule binding two receptor complexes (Shi 2003). The TGF β receptor complexes are normally located in the cytoplasm of cells, but these complexes may migrate to tight intercellular junctions when bound to TGF β (Barrios-Rodiles 2005). Upon binding of TGF β , the TGF β RII receptor phosphorylates the TGF β RI receptor, activating it by switching its conformation to a preferred binding site for receptor regulated SMAD (r-SMAD) substrates (Shi 2003). The latency associated polypeptide (actually the proregion of the TGF β precursor), decorin and α 2-macroglobulin all bind to free TGF β , preventing its receptor binding.

R-smads 2 and 3 are directly phosphorylated by the active TGF β RI's whilst held near the surface membrane by Smad anchor for receptor activation (SARA). This phosphorylation increases their affinity for the co-SMAD, Smad 4; the complex formed is then translocated to the nucleus. Here the complex regulates target genes, approximately 500 of which have been identified either increasing or decreasing their transcription; these responses often depend on cell type and specific cellular conditions (Shi 2003).

1.7.2 TGF β and airway remodelling

TGF β is overexpressed in asthmatic airway epithelium, and is found in higher concentration in the BAL of asthmatics compared to controls, and evidence from animal models that TGF β is fundamentally involved in airways remodelling is widespread (Broide 2008). In various models, including one used by McMillan *et al*, based on OVA sensitisation and provocation, neutralising TGF β using a specific antibody, or via a knockout mouse model of the downstream signalling pathway (Smad3 knockout), resulted in a significant reduction in peribronchial fibrosis, airway smooth muscle proliferation and mucus production without a change in airway inflammation (McMillan 2005). *In vitro* work has demonstrated that TGF β stimulates fibroblasts to produce extracellular matrix proteins and decreases the production of collagenases whilst increasing production of proteins that inhibit tissue inhibitor of metalloprotease (TIMP) (which normally degrades the extracellular matrix) (Wynn 2007).

Although the overwhelming weight of evidence suggests that airway remodelling is TGF β dependent, there is evidence from some studies that airway remodelling may be independent of TGF β , but that airway inflammation is TGF β dependent (Fattouh 2008). Notably this study used house dust mite (HDM) to trigger an allergic model of asthma in Balb/c mice, rather than the more commonly used OVA method, which may have had an impact on the results achieved. The authors administered 10U of HDM extract to each mouse

in the study daily for 5 weeks; this is the same absolute dose (3000 times the dose based on body mass) that most volunteers in our study received on three occasions, 48 hours apart. Such massive doses may well have induced unusual results. Simultaneous with administration of HDM extract whilst anaesthetised, the animals were treated on alternate days with a TGF β neutralising antibody, or the HDM exposure was carried out in SMAD3 knockout mice.

The authors examined subepithelial collagen deposition, ASM thickness and mucus production. All of these measures of remodelling were increased in HDM exposed animals, irrespective of whether they were given anti TGF β or not. TGF β levels in the BAL were decreased by 80% following the neutralising antibody, indicating that the antibody was effective (and was the same antibody used by McMillan *et al* as above). The SMAD3 knockout mice demonstrated remodelling following HDM exposure to the same degree as wild type mice; TGF β blockade did however increase multiple markers of eosinophilic inflammation. Therefore the authors suggested that in HDM exposure TGF β may not play critical role in HDM induced airway remodelling, but it does play a role in regulating eosinophilic inflammation (Fattouh 2008).

This study raises some important questions. Firstly it may be that OVA challenge and HDM challenge trigger some different pathways even if a common core of pathways are activated by both challenges. It is known that OVA challenge and aspergillus challenge result in different outcomes in animals (Zimmerman 2004) and that HDM and grass pollen do in humans (Hatzivlassiou 2010). In a similar study carried out on the same strain of mice as used by Fattouh, but using OVA stimulation, anti TGF β administration did reduce remodelling (McMillan 2005). This difference in results calls into question studies where different allergens have been used to stimulate responses, both in animal models and in humans.

Fattouh *et al* were not the first to show that TGF β independent fibrosis can occur, Kaviratne *et al* have demonstrated that in *schistosoma mansoni* infection, which results in a Th2 inflammatory response and fibrosis, the fibrosis is TGF β independent but IL13 dependent (Kaviratne 2004). However in another mouse model, this time focussing on the lung, IL13 overexpression results in airway eosinophilia and subepithelial fibrosis, but this IL13 response is TGF β mediated (Lee 2004). This model used an IL13 overexpressing mouse construct, and therefore the normal inflammatory milieu is disturbed. In truth there may be various pathways leading to fibrosis, both TGF β dependent and independent. The role of any single molecule in any process is likely to be contextual in terms both of animal species and possibly allergen and dose dependent.

In humans, decreasing eosinophils in asthmatics by using a specific anti IL5 antibody reduced the concentration of TGF β 1 in BAL fluid, reduced the numbers of airway eosinophils expressing mRNA for TGF β 1 and was associated with a reduction in tenascin, lumican and procollagen III in the bronchial mucosal reticular basement membrane (Flood-Page 2003). Notably this reduction in markers of remodelling occurred in the absence of deliberate allergen challenge, though it likely that low level environmental allergen exposure was present throughout.

In humans, the overwhelming body of data suggests that TGF β does mediate airway remodelling in asthma, though the multiple potential sources of the TGF β and the roles of the different TGF β isoforms are still open to investigation

Isoforms of TGF β

TGF β exists in three isoforms, TGF β 1, TGF β 2 and TGF β 3, all of which are expressed in the human respiratory epithelium (Aubert 1994, Coker 1996, Vignola 1997). Previous experiments on asthmatic airways have demonstrated

that TGF β 1 is localised to submucosal and inflammatory cells including eosinophils and macrophages and is also found on structural airway constituents such as fibroblasts, smooth muscle cells and the extracellular matrix. The expression in the epithelium is more variable (Redington 1998, Coker 2001, Ohno 1996). TGF β 1 is elevated at baseline in bronchoalveolar lavage fluid from asthmatics (Redington 1997). TGF β 2 is localised to the respiratory epithelium and is increased in asthmatic patients, in addition, asthmatics demonstrate increased numbers of TGF β 2 positive eosinophils and neutrophils following allergen challenge (Balzar 2005). Although it appears that TGF β 2 levels in BAL from asthmatics are not increased at baseline, they are increased following allergen challenge (Batra 2004, Bottoms 2010). There is little data regarding the final human TGF β isoform, TGF β 3, what evidence there is however, suggests no difference between asthmatics and control groups (Balzar 2005, Torrego 2007).

In order to investigate the role of TGF β in the respiratory epithelium following repeated bronchoconstriction with and without additional inflammation, all TGF β isoforms will be examined in biopsy specimens in this study. As TGF β 2 has been shown to be elevated following allergen challenge, this isoform will initially be examined in BAL fluid and cell culture supernatant.

1.7.3 The Adipokines

The adipokines are a group of hormones including leptin, adiponectin and resistin which were originally described as being released from adipose tissue in mouse models of obesity (Steppan 2001a). Resistin was originally described in mice following treatment with the glitazone group of drugs, which improve glucose tolerance in type II diabetes. Glitazones bind to the peroxisome proliferator activated protein- γ (PPAR γ); a nuclear receptor abundant in fat cells. Binding results in downregulation of a number of genes, one of which is resistin. In humans the Resistin gene is found on chromosome 19 and encodes a

12.5kDa, 92 amino acid protein with a distinctive cysteine rich C terminus (Steppan 2001b).

Expression of resistin is induced during adipocyte differentiation, found in serum, and its concentration increased in genetic and diet induced obesity in mice. Immunoneutralisation improves Type II diabetes and insulin action in mouse models, whilst administration of resistin impairs glucose tolerance and insulin activity in normal mice (Steppan 2001a). Despite these initial findings, resistin and other adipokines have not fulfilled their promise of being fundamental to the development of either obesity or type II diabetes; in humans, plasma resistin levels are elevated in patients with Type II diabetes but are not associated with human insulin resistance or obesity (Youn 2004). Whilst the adipokines were being investigated, it was however found that resistin is involved in Th2 cell mediated inflammation, increasing the expression of IL1, IL6, IL12 and TNF α in an NF- κ B mediated fashion (Silswal 2005).

Not only has resistin been identified as an inflammatory mediator, a family of resistin-like molecules has also been identified in a variety of tissues (Steppan 2001b). These molecules have been subject to various naming conventions as shown in Table 1-1.

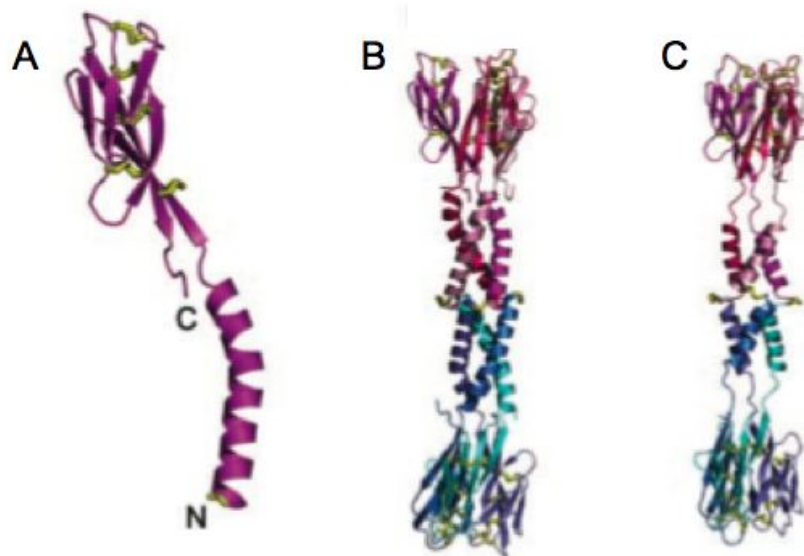
The initial Resistin-like molecules (RELM) were identified in humans and mice by functional genomics based on the unique cysteine rich C terminus of mouse and human resistin (Steppan 2001b). The signature region of the RELMs contains a unique and invariant spacing of the cysteine residues: C-X₁₁-C-X₈-C-X-C-X₃-C-X₁₀-C-X-C-X-C-X₉-CC. Though human RELM α was described by the authors who found the resistin like molecule family, it was later determined that this was in fact human resistin; a human form of RELM α has not been found (Steppan 2001b, Yang 2003).

Table 1-1 Naming conventions for the resistin family of proteins.

Molecule	Gene symbol	Other names	Species
Resistin	RETN	FIZZ3	Hu, mouse
Resistin like alpha	RETNLA	FIZZ1, RELM-alpha, RELM α , hypoxia induced mitogenic factor	Mouse
Resistin like beta	RETNLB	FIZZ2, RELM-beta, RELM β	Hu, mouse
Resistin like gamma	RETNLG	FIZZ3, RELM-gamma, RELM γ	Mouse

Hu – Human. From Teng 2003, Holcomb 2000, Steppan 2001a/b

Figure 1-2 Ribbon diagrams of resistin and resistin like molecule beta (RELM β). The resistin monomer is composed of a carboxy-terminal disulfide-rich globular domain and an amino-terminal helical region (A). These monomers form trimer-dimer hexamers; resistin (B) and RELM β (C). Each trimer is disulphide bonded (shown in yellow) to the associated trimer. From Patel *et al*, Science 2004.



Human resistin and RELM β are soluble proteins which normally exist as trimer-dimers linked by disulphide bonds. Both molecules initially form monomers consisting of a cysteine rich C terminus forming a disulphide rich globular β -sandwich 'head' and a helical 'tail' at the N terminus. The head domain adopts a six-stranded jelly-roll topology and contains two three-stranded all-antiparallel β pleated sheets as shown in Figure 1-2. No human proteins with similar domains have been found in the Protein Data Bank. These monomers form trimers by formation of parallel coil coils of the N terminus tails; these trimers then form trimer-dimers by disulphide bonds as shown in Figure 1-2 (Patel 2004).

Whilst RELM molecules were originally described using mouse resistin as a basis for functional genomics, they were also described, by a different group, following exposure of mice to experimentally induced pulmonary inflammation and named Found in Inflammatory Zone (FIZZ) 1, 2 and 3 (Holcomb 2000). These corresponded to RELM α , RELM β and resistin respectively. The initial studies examined BAL fluid from OVA challenged mice and compared bands on SDS-PAGE to mice not exposed to OVA. The FIZZ1 protein (now named RELM α) was found primarily in the lung, but also in cardiac and skeletal muscle. In the lung, in situ hybridisation demonstrated that RELM α was expressed in the large airways in discrete clusters of epithelial cells at rest, and following OVA challenge, expression increased markedly with uniform expression in bronchial mucosal epithelial cells. Immunohistochemistry demonstrated alveolar type II pneumocytes and alveolar macrophages also expressed the molecule. Similar results were achieved with a house dust mite exposure model (Holcomb 2000). RELM α has also been shown to be induced in the mouse lung epithelial, type II pneumocyte and pulmonary vascular cells by hypoxia, and that it has potent angiogenic and pulmonary vascular constrictive properties mediated by the PI3K pathway (Teng 2003).

Transcript expression profiling of BALB/c mice following exposure to OVA or aspergillus antigen has also revealed increased expression of the RELM

molecules. In this series of experiments, repeated OVA challenge (two intranasal challenges) or repeated aspergillus challenges (3 times a week for 3 weeks) were performed in wild type or STAT6 knockout BALB/c mice. At 13 hours following the initial OVA challenge 57 genes were induced, by 18 hours following the second OVA challenge, 414 genes were induced (Zimmermann 2004). Due to the methodology used, it cannot be determined if the second OVA challenge or longer time interval following the initial challenge lead to the increase in gene expression. Using the same transcript expression techniques, the authors demonstrated the induction of 387 genes following aspergillus stimulation. Only 242 genes were induced with both OVA and aspergillus stimulation, these were termed asthma signature genes. The asthma signature genes included RELM β as well as genes implicated in remodelling such as tenascin, collagen III and collagen IV. Repeating the experiments in STAT6 knockout mice revealed the majority of OVA induced genes to be STAT6 mediated, with only 60 genes being upregulated, whilst in the aspergillus stimulated STAT6 knockout mice 285 genes were upregulated. RELM β was not induced in the STAT6 knockout mice following either OVA or aspergillus stimulation (Zimmermann 2004). The findings imply that RELM β expression is STAT6 mediated, and may require repeated antigen stimulation to trigger its production.

Work in cultured bone marrow cells has shown that RELM α is rapidly and significantly upregulated (increase detectable at 1 hour and up to 10,000 times increase at 24 hours) by both IL4 and IL13, whilst RELM β expression was not affected by either IL4 or IL13. This group also suggested that there were no identifiable STAT6 binding sites in the RELM β promoter, whilst these were present in the RELM α gene (Stutz 2003).

In whole mouse experiments performed by the same group RELM α and RELM β were upregulated after a single allergen challenge in homogenised lung tissue, suggesting that repeated challenge is not required. At 6 hours after challenge

RELM α mRNA was increased by 34x whilst RELM β mRNA was not initially detectable, but after challenge went up by 58x. Levels of resistin (FIZZ3 in this model) were ten times lower than RELM α in controls and was slightly reduced following allergen challenge. The absolute levels of RELM β were 1000x less than RELM α in allergen challenged animals. The rise in both RELM α and RELM β following the same allergen challenge was abrogated in STAT6 knockout mice, again implying that both RELM α and RELM β are STAT6 mediated, despite only RELM α being IL4 / IL13 mediated in the cultured cells (Stutz 2003). The authors hypothesised that RELM β expression was dependent on another intermediate molecule which itself was STAT6 mediated.

Follow up work by the same group which performed the transcript expression profiling, using the same mouse model, has given us more insights into the pathways mediated by RELM β (Mishra 2007). Again using BALB/c mice, OVA was administered by inhalation at days 24 and 27 following sensitisation, whilst aspergillus antigen was administered three times weekly for three weeks. All sampling was performed 18-20 hours following the last challenge. RELM β mRNA measured by qPCR was induced by both stimuli – in keeping with the previous transcript expression profiling work (Zimmermann 2004). RNA production measured by Northern blotting confirmed these results, with no RNA detectable prior to challenge. Resistin levels were not increased by either challenge.

Using in situ hybridisation, RELM β RNA was not detectable prior to challenge in lung histological sections, but following OVA or aspergillus exposure was found in epithelial cells and infiltrative mononuclear cells surrounding blood vessels and airways. In IL13 and IL13+IL4 knockout mice there was no rise in RELM β following allergen challenge. When IL4 or IL13 were administered intratracheally RELM β mRNA was induced, though not in STAT6 knockout mice. These findings imply that RELM β induction is mediated via an IL4 / IL13

and STAT6 pathway, in contrast to the cell culture work described above (Stutz 2003).

RELM β recombinant protein administered tracheally (10 μ g per administration – 7 doses given on alternate days) induced both macrophage accumulation in BAL and perivascular and peribronchiolar collagen deposition. Total lung collagen increased from 52 μ g/mg to 105 μ g/mg following RELM β administration. There was also a 10% increase in PAS positive goblet cells.

The same group produced RELM β knockout mice which suffered no detectable phenotypic abnormalities to wild type mice prior to OVA or allergen challenge; following such challenges there was the same degree of eosinophilic infiltration into the lung parenchyma and BAL as wild type mice, but a 32% reduction in the amount of collagen deposition that occurred.

From these results it was clear that in the mouse model used, RELM β played a role in remodelling and collagen deposition following experimental allergen challenge. This might have implied that RELM β would stimulate fibroblasts to proliferate – this however was not the case, though RELM β did induce fibroblast motility in a dose dependent manner down to concentrations as low as 10nM. Similar results with human fibroblasts were achieved using human recombinant RELM β (Mishra 2007).

Therefore this group demonstrated that RELM β is induced by allergen and the Th2 cytokines IL4 and IL13 and regulated by STAT6. It directly induces collagen deposition and is necessary for the fibrotic response to allergen. It is a proinflammatory cytokine induced by allergen exposure and has an effect on both human and murine fibroblasts. It appears that RELM β is one of a family of mediators that act downstream of the immune response, amplify the inflammatory response and appear to mediate fibrosis in mouse models of

asthma; and may have a similarly important role in human asthma and remodelling (Homer 2007, Mishra 2007).

Currently no work has been published examining RELM β in humans either under baseline conditions, or following allergen challenge

1.7.4 Osteopontin

Osteopontin is a acidic phosphorylated glycoprotein that contains the arginine-glycine-aspartate (RGD) integrin binding domain common to matrix proteins. It was initially described in bone as a bridging protein, resulting in its being named osteo from bone and pontin from Latin 'pons' for bridge (Senger 1979).

As part of its function, bone is constantly exposed to weight bearing or muscular tension, and bone growth is influenced by the mechanical stress placed upon it. In a human osteoblast cell line and in primary osteoblasts from mice, OPN has been shown to be upregulated following compressive stress applied apically to submerged cultured cells at 0.3Hz with a maximal pressure of 13kPa (Klein-Nulend 1997). Though these pressures are much greater than the theoretical pressures generated in the airway in response to bronchoconstriction, which are thought to be around 15 to 30 cm of water (or 1.4 to 2.8kPa) (Wiggs 1997) these data demonstrate that OPN is upregulated by mechanical force in at least one cell type.

Although initially described in bone, OPN has been found in many other cell types and been shown to have both pro and anti-inflammatory effects. Its proinflammatory effects include the support of adhesion and modulation of function of T cells, monocytes and macrophages, as well as being chemotactic to macrophages. It also increases the expression of MMP1 and is chemotactic to lung fibroblasts. Its anti-inflammatory actions include the inhibition of nitric oxide (NO) production in the epithelial cells of the kidney; NO has itself been

shown to induce the production of OPN, hence providing an auto regulatory loop (Denhardt 2001).

OPN has been shown to bind to integrin receptors α_v and β_1 , allowing OPN to mediate cell-matrix and possibly cell-cell interactions; OPN can be cleaved by thrombin, resulting in exposure of a cryptic binding site, which has been shown via interaction with $\alpha_v\beta_3$ receptors, mediates endothelial cell migration during angiogenesis; OPN following cleavage by thrombin is chemotactic to endothelial cells and may promote new vessel formation (Senger 1996, Puxeddu 2009).

In a mouse model of idiopathic pulmonary fibrosis (IPF) (triggered by bleomycin administration) OPN was found to be elevated and produced by alveolar macrophages (Takahashi 2001). *In vitro* experiments conducted by the same group demonstrated that OPN increased fibroblast migration, adhesion and proliferation all of which were blocked by a GRGDS peptide or an α_v antibody; bleomycin induced lung injury was attenuated by the administration of α_v antibody (Takahashi 2001). The role of OPN in pulmonary fibrosis was supported by work using microarrays in normal and fibrotic lung tissue showing that the expression of OPN was increased in the IPF lung; in addition, concentrations of OPN are increased in BAL fluid from IPF patients compared to controls. *In vitro* work accompanying these findings supported the initial work showing that OPN is chemotactic to fibroblasts, and in addition also showed that it is chemotactic for epithelial cells. OPN also induced an increase in collagen gene expression in a fibroblast cell line. All of these effects were blocked by antibodies to $\alpha_v\beta_3$ integrin or by the peptide GRGDS (Pardo 2005).

Osteopontin has been shown to be expressed in human eosinophils (Puxeddu 2009), and has also been identified as a chemotactic factor for eosinophils *in vitro* (Takahashi 2009). In mouse models of asthma, OPN is upregulated in airway epithelial cells (Takahashi 2001) and induces airway remodelling and

lung fibroblast activation (Kohan 2009) whilst a mouse knockout to OPN protects against both airway hyperreactivity and airway remodelling following OVA challenge (Simoes 2009).

Osteopontin has not been examined in humans following allergen challenge.

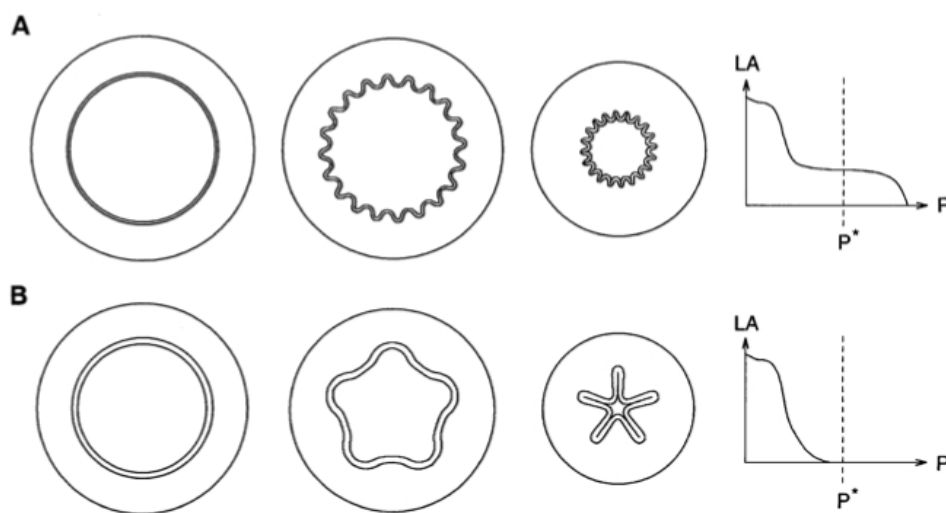
1.8 Clinical impact of airways remodelling

Asthma is characterised by both an increased sensitivity of the airways to trigger contraction of the smooth muscle (hypersensitivity) and an increased narrowing following that contraction (airways hyperresponsiveness (AHR)). In normal individuals where airway narrowing is induced experimentally, the degree of narrowing reaches a moderate level and does not progress further despite continued stimulation (Moore 1996).

AHR is related to remodelling within the asthmatic airway. Any increase in the thickness of the airway wall luminal to the smooth muscle mass has been hypothesised to result in a greater restriction in airway lumen from the same degree of smooth muscle constriction. Also, remodelled airways in asthma have a larger mass of ASM, which may contribute to the greater degree of airways hyperreactivity. AHR to methacholine has however been found to be strongly negatively correlated with airway wall thickness as measured by helical CT or endobronchial ultrasound (Park 1997, Park 2006, Niimi 2003). In these studies, AHR to methacholine did not correlate with sputum eosinophils, though airway hypersensitivity did, whilst airway reactivity was negatively correlated with airway wall thickness. An increase in ASM mass in larger but not small airways is a feature of fatal asthma, which is not present in non fatal asthma, and recent work has demonstrated that therapeutic destruction of the ASM by bronchial thermoplasty appears to reduce AHR (Carroll 1993, Fixman 2007, Pavord 2007).

Increased matrix proteins, such as collagens could have significant effects on the mechanical properties of the airway wall. When the airway constricts following ASM contraction, there is a buckling of the epithelial surface and RBM, similar mechanical impact has been shown following constriction of blood vessels and the Eustachian tube (Wiggs 1997). Mathematical modelling of the airway has been established using a bilayer model; in this model the inner layer represents the RBM and epithelium and the outer represents the submucosal and mucosal regions luminal to the ASM (Figure 1-3). Using this model it has been shown that increasing the thickness of the inner layer (RBM and epithelium) results in fewer but larger folds in the airway lumen, and subsequent earlier airway narrowing. Hence it is suggested that airway remodelling with thickening and decreasing distensibility of the RBM contributes to symptomatic AHR and bronchoconstriction with the same level of mechanical force generated by the ASM (Wiggs 1997). Unlike mathematical modelling, which utilizes the concept of airway force generation and maximal contractive pressure, clinical measures of AHR most commonly include FEV₁ as a surrogate marker of degree of bronchoconstriction.

Figure 1-3 Schematic diagram of the buckling effect of airway smooth muscle constriction on the airway epithelium and basement membrane. With a thin basement membrane (A) as smooth muscle constricts the folds can push against each other, maintaining the airway lumen. With a thick basement membrane (B) the larger but fewer folds result in greater airway narrowing, and may result in airway occlusion at the point of maximal contractive pressure (P^*). From Wiggs *et al* J Appl Phys 1997.



1.9 Airway remodelling - pathological, or maligned and misunderstood?

Airway remodelling up to now has been referred to as a purely maladaptive pathological response seen in the airways of patients with asthma. This may not be the case; airway remodelling, or at least aspects of it, may be a beneficial adaptive response to repeated airway constriction. In the mathematical model above, the dominant influence is the stiff subepithelial collagen layer; in order to generate the model, the basement membrane was considered as an inextensible membrane - one which was flexible but which

could not be compressed or stretched (akin to a bicycle chain). With this assumption as to the character of the basement membrane, any compression of the airway must result in epithelial folding, however the RBM may not be as inextensible as thought when that model was constructed. In an elegant study of resected human lung tissue, McParland *et al* demonstrate that the basement membrane is capable of being stretched by approximately 50% following inflation of the lungs with 21cm of water pressure, compared to that when resected specimens were not inflated. This suggests that the RBM is in fact elastic and the epithelium resting on its surface is likely to undergo compressive and stretching forces both during the mechanics of normal breathing, but also during episodes of bronchoconstriction (McParland 2004). Such compressive forces present during bronchoconstriction would give a differential stimulus to the airways of patients with asthma, as bronchoconstriction is pathognomonic of an asthma attack. The physical stresses from bronchoconstriction might be triggering responses from the epithelium and underlying tissue only in asthma because only in asthma is there such a degree of bronchoconstriction; the changes seen in asthma could be a 'normal' response to that degree of mechanical stress, which is only seen in asthma as a response to bronchoconstriction.

Theoretical stresses in the airway wall (epithelium and lamina propria) during bronchoconstriction are relatively high (around 3kPa) compared to stresses in blood vessels (around 1.5kPa) that are known to elicit responses from the blood vessel endothelium (Adamo 2009). If individual cells in the airway epithelium responded to mechanical stress by triggering production of a greater quantity of, or less distensible, supporting fibres, thus protecting themselves from ongoing mechanical stress, this would be an understandable physiological response. If in doing so, the thickened RBM resulted in more airway occlusion for the same degree of airway smooth muscle constriction and hence more symptoms, the response would initially be interpreted as maladaptive, whereas on an individual cell level it may be physiological and warranted.

Remodelling could also be beneficial if it resulted in the deposition of a less distensible collagen sheath surrounding the ASM, which therefore restricted the maximal muscle shortening. In addition, epithelial metaplasia with increased mucus glands and hence mucus secretion may protect the airway from inhaled toxins and excessive dehydration (McParland 2003). It is known that airway drying leads to osmotic gradients across the epithelium, which in turn result in mast cell degranulation and bronchoconstriction (Brannon 2003). Adaptation of the epithelium to produce more mucus could prevent such drying and osmotic shifts, hence potentially lessening the frequency of bronchoconstriction.

Although asthma has a faster rate of FEV₁ fall than the general population in adults once maximal lung function has been achieved (38 vs 22 ml / year), and this has been attributed to remodelling, there is as yet no specific evidence to support this assertion (Lange 1998, Fixman 2007).

1.10 Causes of airway remodelling

Various models of the causes of remodelling in asthma exist, specifically related to the relationship between airway inflammation and the remodelling described briefly above. These models may be considered in three broad groups. The first considers that the remodelling events are purely secondary to eosinophilic inflammation, which would suggest that airway remodelling in asthma would only be present late in the disease, after sufficient cycles of inflammatory damage had been established to trigger the remodelling events. The second model proposes that eosinophilic inflammation and remodelling are processes that occur in parallel, possibly driven by the same or similar triggers, which would predict that both remodelling and eosinophilic inflammation would be present together in most or all circumstances. The third model is that eosinophilic inflammation is not a primary abnormality in the asthmatic airway, rather the prime disorder is of abnormal airway repair mechanisms which lead

to remodelling, and any inflammation seen is a secondary or possibly even entirely unlinked event.

In adults with asthma, the presence of airways remodelling and eosinophilic inflammation almost invariably coexist, which is also the case in children aged 6 - 16 years of age. In a study of children in this age group with difficult asthma, compared to both control adult and paediatric groups and mild (British Thoracic Society (BTS) step 1) and life threatening asthma groups there was no difference in the RBM thickness between any of the asthma groups; all demonstrated RBM thickening compared to non asthmatic controls. There was no association with age, symptom duration, lung function or concurrent eosinophilic inflammation (Payne 2003). Another paediatric study examined airway remodelling markers and eosinophilic infiltration before a diagnosis of asthma had been made in preschool children, here they found the presence of airway remodelling in terms of thickened RBM, and also more eosinophils in the bronchial mucosa in children (aged 1.2 - 11.7) who went on to be diagnosed with asthma compared to those who did not (Pohunek 2005). Further work has shown that eosinophilic inflammation and RBM thickening are not present in infant (mean age 12 months) wheezers, but are both present in wheezy preschool children (age 1 to 3 years) (Sagani 2005 and 2007). This would suggest that the paradigm of inflammation followed by remodelling either has an extremely short time scale between the initiation of inflammation and remodelling, or such a sequence is not fundamental to the presence of remodelling. In the absence of true longitudinal data in single individuals, snapshots in time, as obtained in the above studies, are likely to represent the best data realistically available, and these demonstrate that airway remodelling and airway inflammation are detectable at the same time points.

1.11 Eosinophils in asthma and remodelling

The eosinophil has been regarded as the fundamental inflammatory cell in the pathology of asthma since William Osler described asthma as an inflammatory condition with 'asthma crystals' (later identified as eosinophilic granulocytes) in the sputum. Despite the eosinophil being recognised in the airway for over a century, its exact role has yet to be elucidated (Diamant 2007, Kariyawasam 2007). There is a body of evidence that is pointing towards a role, possibly a fundamental role, for the eosinophil in the remodelling process that occurs in asthma, though this remains controversial (Choe 2003). The eosinophil is a bone marrow derived white cell, comprising less than 5% of circulating white cells in normal individuals. Eosinophils differentiate from myeloid precursors in response to stimulation with IL3 and granulocyte monocyte cell stimulating factor (GM-CSF) whilst IL5 is the signal required for terminal differentiation to mature eosinophils from eosinophil committed precursors (Leckie 2000, Floodpage 2003). This terminal differentiation may take place both within the bone marrow, and within the bronchial mucosa (Menzies-Gow 2007).

Eosinophils are thought to play a primary role in host defence against parasitic infection; they contain granules that contain a variety of cytotoxic substances including major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP). Release of these intracellular granules may result in significant local tissue damage, and may be readily measured to make an assessment of eosinophil recruitment and activation within the airway.

The exact role of the eosinophil in asthma is unknown, but eosinophils are virtually pathognomonic of the disease and are present early in the disease, prior to the onset of symptoms in children from the age of 1 year. Notably they are absent in children with recurrent wheeze prior to this age (Saglani 2005 and 2007). It is known that allergen challenge results in eosinophils migrating from the bone marrow to the lung, and that such a migration may be triggered by

inhalation of IL5 in normal individuals, but not asthmatics (Menzies-Gow 2007). Eosinophil derived proteins including MBP and EPO when instilled into the trachea in primates, result in bronchoconstriction and AHR (Gundel 1991). The presence of eosinophils results in AHR by a variety of mechanisms; MBP binds to inhibitory muscarinic receptors (M2) on parasympathetic nerves, resulting in increased acetylcholine release and AHR, in addition, eosinophils are a source of the leukotrienes D4 and E4 which induce mucus hypersecretion as well as oedema and AHR in addition to bronchoconstriction (Adelroth 1986).

Recently clinical studies of anti IL5 antibodies have demonstrated a reduction in exacerbation frequency and an improvement in asthma related quality of life (AQLQ) scores in severe asthmatics (Haldar 2009, Nair 2009). From this it is clear that eosinophils are likely to be fundamental to the pathogenesis of asthma, but their role in asthma induced remodelling is less clear. That the eosinophil may be fundamental to airway remodelling has biological plausibility; eosinophils produce not only the proteins mentioned above, but also others, which they secrete into the airway microenvironment. These include the potent fibrogenic molecule, transforming growth factor beta (TGF β) which not only is chemotactic for fibroblasts, but also induces the differentiation of fibroblasts into myofibroblasts.

Stimulation of fibroblasts with TGF β results in rapid collagen I and III upregulation, and hence provides a pathway whereby an increase in eosinophils within the airway may result in collagen deposition and remodelling. Notably, many other cells in the airway also secrete TGF β , including epithelial cells, macrophages, mast cells and lymphocytes, so even if eosinophils contribute to airway remodelling, they may not be necessary for it to occur (Tschumperlin 2003, Kariyawasam 2007).

1.11.1 Eosinophil depletion studies

Early convincing evidence for the role of the eosinophil in remodelling came in eosinophil depletion studies, performed using anti IL5 antibodies which depleted both circulating and bronchial eosinophils (Flood-Page 2003). A humanised anti IL5 monoclonal antibody (mepolizumab) was administered intravenously in atopic human volunteers monthly for 3 doses, with bronchoscopy and biopsy performed before and after the infusions. Mepolizumab resulted in a significant reduction in the numbers of eosinophils in the airways and this correlated strongly with a concomitant reduction in the basement membrane deposition of a variety of structural proteins including tenascin, lumican, and procollagen III. Tenascin levels were reduced to levels seen in normal individuals. There was also a reduction in the concentration of TGF β 1 in BAL fluid (Flood-Page 2003).

Mepolizumab does not have an effect either on responses following allergen challenge (Leckie 2000) or on clinical symptoms in initial studies (Flood-Page 2007), though it does reduce exacerbation frequency, allows steroid sparing and improved AQLQ scores in more recent trials (Halдар 2009, Nair 2009). This does not provide the only evidence for the eosinophil being involved in the control of ECM matrix deposition; patients with eosinophilic bronchitis have no AHR, but do demonstrate thickening of the epithelial basement membrane (Brightling 2003). Eosinophilic oesophagitis is also associated with increased ECM matrix deposition (Rothernberg 2009). Early clinical investigations into the effects of mepolizumab, where a single intravenous dose was given, produced long term suppression of circulating eosinophils and degree of sputum eosinophilia following allergen challenge, however it did not affect the presence or amplitude of the late asthmatic reaction (LAR) or AHR following allergen challenge. This would imply that eosinophils are not a prerequisite to the development of the LAR or AHR, but it is known that they are generally associated with it (Leckie 2000). Other studies have also shown a dissociation of airway inflammation and AHR (Bryan 2000).

1.11.2 Animal models of remodelling implicating the eosinophil

Animal models of asthma have also suggested that the eosinophil is fundamental to the remodelling pathway, though the relevance of animal models in the investigation of asthma is controversial (Krug 2008, Martin 2006, Kumar 2002, Persson 2002). Investigations in an IL5 knockout mouse have shown resistance to remodelling despite repeated allergen challenge twice weekly for 3 months, demonstrating decreased collagen III and IV deposition as well as total lung collagen content and less peribronchial smooth muscle; they also demonstrate a reduction in TGF β 1 expressing eosinophils (Youn 2004). Transgenic mice engineered to constitutively express IL5 in bronchial epithelium show accumulation of peribronchial eosinophils, goblet cell hyperplasia, collagen deposition and epithelial hypertrophy (Lee 1997).

The transcription factor GATA-1 is expressed in many haemopoietic lineages, including eosinophils. Specific deletion of a high affinity GATA binding site in the GATA-1 promoter can be engineered to produce mice lacking the eosinophil lineage, but normal in all other respects (Yu 2002, McMillan 2004). Repeated OVA challenge in such mice results in no detectable eosinophils in the bone marrow or airway. Despite this absence of eosinophils there was no change in Th2 cytokines levels, AHR to methacholine or mucus secretion compared to wild type mice. With respect to collagen deposition, the GATA promoter deletion lead to decreased, but not absent, collagen deposition, ASM proliferation and hyperplasia. The findings appeared to be independent of the actions of TGF β 1, where no differences in expression were found (Humbles 2004). These findings are in contrast to the IL5 knockout studies above, where eosinophils were the predominant source of TGF β 1. Other eosinophil depletion studies have also contradicted the GATA deletion work, showing that epithelial cell hypertrophy and mucus cell metaplasia were also reduced (Lee 2004). These fundamental differences between animal models, which do not provide good correlation with human asthma, reinforce the requirement for good human

studies to be performed (Bush 2008, Zosky 2008, Krug 2008). Further evidence for the importance of eosinophils and TGF β come from a study that examined the effects of an antibody against CD300a (which inhibits mast cell and eosinophil activation). Using this antibody in a chronic animal model of asthma, the authors demonstrated that remodelling could be reversed by intranasal administration of the antibody. Bronchoalveolar lavage (BAL) lymphocytes and eosinophils were reduced to normal levels by 60 days, and there was also a reduction in BAL TGF β and IL5, again contrasting with the GATA promoter deletion findings. Airway remodelling in terms of RBM thickness, ASM mass and mucus production were all reduced. These results suggested that although eosinophils were a major source of TGF β they were not the only source, but did demonstrate that remodelling was reversible. The antibody was not specific to eosinophils however, also reducing lymphocyte and mast cell numbers (Munitz 2006).

1.11.3 Non TGF β mediated eosinophil pathways in remodelling

The eosinophil is implicated in airway remodelling by pathways other than those involving TGF β . Eosinophil MBP is directly cytotoxic to the airway epithelium, resulting in epithelial detachment and ciliary dysfunction *in vitro*. These effects may result in activation of the epithelium, which itself is a potent source of TGF β and other cytokines, though mechanical damage of the epithelium can also result in epithelial activation in the same system (Zhang 1999). ECP also may have a role in the submucosal deposition of ECM proteins by the inhibition of heparanases, which result in decreased proteoglycan degradation in fibroblasts, leading to proteoglycan accumulation within the cell layer in a culture system. There was however no increase in collagen or in the numbers of fibroblasts in this model (Hernnas 1992).

Eosinophils have also been implicated in the remodelling process by their production of heparin binding epidermal growth factor (HB-EGF), matrix

metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF). All of these substances, or the balance between them is known to effect ECM matrix deposition and angiogenesis (Kariyawasam 2007).

Finally, using a three dimensional tissue culture model of the airway, using cocultured epithelial cells and fibroblasts subjected to compressive force in the presence and absence of eosinophils, Choe *et al* have demonstrated that epithelial thickening occurs when a combination of mechanical strain and eosinophils are present. There was no epithelial thickening when either stress or eosinophils were present alone, notably however in this model there were no standard measures of airway remodelling such as collagen deposition (Choe 2003). In addition, similar experiments performed by the same group in the absence of eosinophils but with both epithelium and fibroblasts present, result in increased collagen deposition, which the authors attributed to epithelial control of the remodelling process (Choe 2006).

1.12 Role of the epithelium

As the airway epithelium provides the interface between the wider environment and lung tissue; epithelial pathology may be fundamental in asthma. The airway epithelium is routinely exposed to the wide gamut of inhaled substances experienced by asthmatic and normal individuals on a daily basis, including allergens, airbourne pollutants, infectious agents, and drugs used in the diagnosis and treatment of asthma. For these provocants to enter the airway tissue they must pass through the epithelium, and only then interact with the majority of immune or inflammatory cells; some immune cells, such as dendritic cells appear to sample environmental antigen by penetrating epithelial tight junctions (Swindle 2009). However, this entry of substances into the airway may not be required for asthma pathology if the epithelium itself, rather than forming a simple barrier function, is able to mediate and modulate airway responses to

external stimuli. Epithelial cells express toll like receptors (TLR's) which respond to various stimuli including bacterial lipoteichoic acid and peptidoglycans (TLR2), hyperoxia and dsRNA (TLR3) (Murray 2008) ssRNA (TLR7) (Camateros 2007), guanidine rich oligonucleotides and ssRNA (TLR8) (Camateros 2007) and CpG DNA motifs in viruses and bacteria (TLR9). Blocking TLR7 and 8 using a specific blocking antibody resulted in reduced remodelling following OVA stimulation in a brown Norway rat model of asthma (Camateros 2007). Stimulation of TLR's 2,3,8 and 9 on epithelial cells results in release of thymic stromal lymphopoietin (TSLP) (Allakhverdi 2007). TSLP then acts at multiple sites including at a receptor on dendritic cells (DC) resulting in the increased expression of CD40, OX40 and CD80, and thus to enhanced Th2 polarisation. Mast cells also express TSLP receptors and are an important source of Th2 cytokines including IL5,6,10,13 and GM-CSF. This pathway gives a T cell independent route for the release of cytokines normally thought to be Th2 cell in origin, and may explain how mice that are deficient in T cells and IgE express an asthma phenotype when overexpressing TSLP (Holgate 2007, Lui 2007). This also gives a mechanism by which the epithelium may mediate the asthma phenotype, without a requirement for environmental stimuli to cross the epithelial barrier.

1.13 Airway response to mechanical stress

The epithelium may also respond to physical stimulation, rather than responding to the presence or absence of stimulator molecules. *In utero* the progenitor lungs undergo rapid proliferation and orchestrate and control continuous modelling (rather than remodelling) of the extracellular matrix.

The mesoderm differentiates into smooth muscle which wraps around the epithelium, and even *in utero*, smooth muscle contraction against secreted pulmonary fluid results in substantial compressive forces on the airway and a transpulmonary pressure gradient (Tschumperlin 2006). During lung

development there is a significant contribution to control of growth by mechanical forces; where lung distension occurs *in utero*, lung hypoplasia results, with fewer airway generations forming. In experimental conditions, if the foetal trachea is occluded the lung becomes hyperplastic; these responses appear dependent on lung volumes, rather than on lung pressures, suggesting that changes in cellular tissue deformation are the prime signalling transduction pathway (Tschumperlin 2006). *In vitro* stretch experiments have been performed by several groups, mostly stretching human lung fibroblasts from various stages of embryonic development on a variety of substrates and at a variety of mechanical stimuli (varying frequency, magnitude and duration of stretch). These experiments have, not surprisingly, had a wide variety of outcomes, but have demonstrated that human lung fibroblasts respond to mechanical stimuli (Mascarenhas 2004, Mohammed 2007, Thomas 2006). When cocultured with bronchial epithelial cells, fibroblasts respond to intermittent mechanical stretch with increased synthesis of collagens, biglycans and proteoglycans, suggesting that mechanical stretch provides significant regulation of the growth of the lung and deposition of ECM during lung development (Tschumperlin 2006).

In the transition from foetus to adult, lung water is removed and cyclical stretch of the lung occurs due to the function of respiration. Until this point normal and asthmatic individuals have been exposed to the same lung mechanics. In infancy however, wheeze may occur, indicating airway constriction, rather than airway stretch. In early childhood wheezing there is no apparent remodelling or inflammation, but this develops at some point after the first year of life (as above). No matter what is triggering the bronchoconstriction, could the epithelium detect and respond to mechanical forces that result from bronchoconstriction?

It has been demonstrated that mechanical distension induces alterations in mRNA of proteins involved in remodelling; mechanical ventilation of isolated

rabbit lungs for 4 hours with high positive end expiratory pressure (PEEP) increases procollagen III, IV, TGF β 1, FGF and fibronectin protein production, whilst mechanical ventilation of rat lungs with high volumes for just 30 minutes results in increased transcription of 10 different genes including procollagen IV, fibronectin and laminin. Further examination by immunohistochemistry showed 4 of the increased gene products to be localised to the bronchial epithelium. (Berg 1997, Parker 1997). The stretching forces applied in these sets of experiments demonstrate that the bronchial epithelium responds to mechanical stretch following birth, but these conditions are artificial and related to mechanical ventilation. In the abnormal physiology of asthma, the forces are likely to be more complex and based on a compressive force in the airway wall.

1.14 Epithelial response to compressive stress

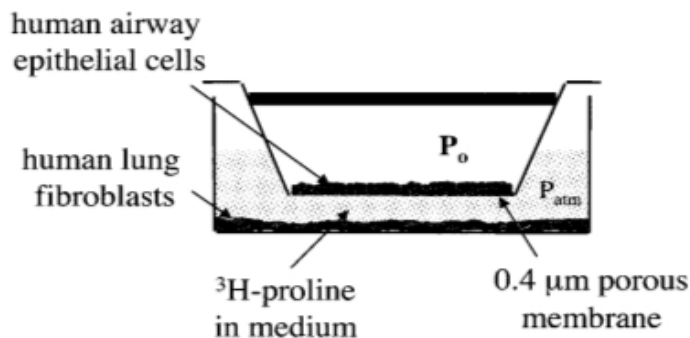
Various *in vitro* models have been developed and used to address whether the bronchial epithelium may respond to compressive stress such as may result from bronchoconstriction. Initially Ressler and colleagues examined rat tracheal epithelial cells and compressed them using an apical increase in air pressure to generate a transmembrane pressure. They demonstrated that early growth response-1 (Egr-1), endothelin-1 (ET-1) and TGF β 1 genes were all upregulated, as well as Egr-1 protein being increased (Ressler 2000). This work was repeated with some modification by the same group using human bronchial epithelial cells, demonstrating again the release of ET-1, and on this occasion ET-2 as well as TGF β 2 from air liquid interface (ALI) cultures of human bronchial epithelial cells. Although gene transcription for the endothelins increased, the TGF β 2 signalling was by release of preformed cell stores in its active form, rather than by increased transcription. ET and TGF β were released independently of each other in response to mechanical stress as demonstrated by blocking antibodies to each. Only short periods of stress (minimum of 1 hour) were sufficient to commit the cells to induce signalling. They also demonstrated that ET and TGF β 2 increase the fibrotic response of fibroblasts both individually

and synergistically but the TGF β is a more potent stimulator of fibrotic protein synthesis than ET (Tschumperlin 2003).

Such responses from epithelial cells infer that remodelling responses might be triggered by increases in transmembrane pressures across the epithelium, however they do not demonstrate that epithelial cells can orchestrate a response from subepithelial fibroblasts to modulate RBM thickness. In order to do this there would have to be a paracrine communication between epithelial cells and fibroblasts. Using an elegant coculture method where epithelial cells above an incompressible but porous membrane were cultured with fibroblasts below the membrane, and a transmembrane pressure applied only to the epithelial cells (shown in Figure 1-4), Swartz *et al* have demonstrated that the fibroblasts increased their production, in a time and pressure dependent manner, of collagen I, III and IV. The epithelial cells increased their production of fibronectin, and demonstrated an increased MMP9/TIMP1 ratio. This emulation of the remodelling process *in vitro* was obtained in the absence of any inflammatory cells (Swartz 2001).

In addition to modulating remodelling, epithelial mechanical stress has been shown recently to alter the barrier function of the epithelium, resulting in a disruption of tight junctions and an increase in the transduction of lentivirus across the epithelial surface (Tomei 2008), though examination of barrier function using FITC Dextran following epithelial cell compression shows no such increase in permeability (Park 2010).

Figure 1-4 Schematic diagram of coculture model of epithelial constriction leading to fibroblast response. P_o indicates pressure above atmospheric. From Swartz *et al* Proc Natl Acad Sci 2001.



1.15 Epithelial signal transduction of mechanical compression

These data show that the epithelium is capable of detecting and responding to mechanical stress, however the mechanism underlying the signal transduction of the pressure to the cell has not been approached.

1.15.1 Mechanochemical signal transduction;

Using ALI cultures of human bronchial epithelial cells, Tschumperlin *et al* have demonstrated that lateral stress on these cells shrinks the intercellular space whilst cell volumes themselves remain constant. This loss of intercellular fluid results in MMP dependent shedding of Heparin-binding epidermal growth factor (HBEGF) from one cell binding to the EGFR on the adjacent cell, with resultant

EGFR ErbB1 phosphorylation within 5-20 minutes of compression, whilst phosphorylation of the EGFR was decreased in a dose dependent manner by HBEGF antibodies (Tschumperlin 2004). In this model, there is no need for force dependent biochemical processes within the cell or cell membrane, simply reducing the intercellular volume is sufficient to induce signal transduction. Extending this work from cell culture into an *ex vivo* whole tissue model using isolated mouse trachea, Tschumperlin *et al* demonstrated that methacholine application to the isolated tissue resulted in phosphorylation of the EGFR. This result was only found when mechanical stress occurred, as when bronchoconstriction was abrogated by pre administration of isoproterenol no EGFR phosphorylation was detected. This suggests that the bronchoconstriction is the fundamental factor, rather than a direct chemical effect following the administration of methacholine (Tschumperlin 2004).

Other groups have performed similar studies using three dimensional coculture models examining the effects lateral compressive strain of 10 and 30% at either 1 or 60 cycles per hour (rather than apical pneumatic) force (Choe 2003). These models have demonstrated similar findings of increased collagen I, III and IV production from fibroblasts (which were exposed to compressive stress along with the epithelium in this model). Collagen III in non strained samples was uniformly distributed, whilst in strained samples was greatest just under epithelium, indicating a concentration gradient from the epithelial surface. Collagen IV was found localised around fibroblasts and under epithelium, though the spatial gradient was less than the collagen III. Fibroblast only strain cultures showed increased collagen III deposition, but to a much lesser degree than the epithelial and fibroblast cocultures. Fibroblast only cultures showed no change in Collagen IV deposition (Choe 2006). These data together suggest that the epithelium is receptive to mechanical (especially compressive) stress and mediates ECM deposition following such stress.

Another aspect of airway remodelling in asthma is the metaplasia of the epithelium towards a more mucus producing phenotype. Recently it has been shown that repeated apical stress in human bronchial epithelial ALI cultures results in increased immunofluorescence staining for the mucin MUC5AC as well as increased MUC5AC gene expression and protein production. Apical force was applied for as little as 10 minutes per day for 14 days, with changes visible by 7 days. Treatment with EGFR kinase inhibitor reduced but did not completely abrogate the response, but full abrogation was achieved with anti TGF β 2, either alone or combination with anti EGFR kinase. This is the first indication that compressive stress alone may alter the cellular composition of the airway (Park 2009). From the method used in this series of experiments, it is unknown if the multiple stimuli used were required for a response, or if the metaplastic response would have occurred following a single stimulus, but would have taken at least 7 days to be appreciable to the techniques used for assessment. In addition, the chitinase like protein YKL-40 which has been shown to be associated with asthma and airway remodelling has also been shown to be upregulated in response to compressive stress *in vitro* (Park 2010).

No examination of the effect of repeated bronchoconstriction in the human airway has been performed, in fact previous work examining the effect of allergen induced airways remodelling has used methacholine alongside allergen to examine concomitant airways hyperreactivity, and hence may have confounded any results that have been obtained (Kariyasam 2007, Phipps 2004).

1.16 Project rationale

In order to assess whether eosinophil recruitment or mechanical stress on the airway, or both, may induce airway remodelling, we aim to assess the impact of each in human volunteers. As a single bronchoconstriction event may not be sufficiently stimulating to induce measurable remodelling changes, the protocol will involve repeated allergen challenge or methacholine induced bronchoconstriction.

We hypothesise that repeated bronchoconstriction will induce an airway remodelling response and this will be independent of induced eosinophilic airway inflammation.

In order to test this hypothesis using human volunteers, initially a repeated challenge model will be established with allergen, methacholine and appropriate controls. The challenged volunteers will then be assessed for change in airway eosinophilic inflammation and remodelling of the airways.

Chapter 2 Materials and Methods

2.1 Patient recruitment and selection.

In vivo study

Subjects with a history of physician diagnosed atopic asthma were recruited by advertisement and using the database of volunteers held by the respiratory department. Protocols for the study were approved by the Southampton and South West Hampshire Research Ethics Committee (approval number 08/H0502/6), and all subjects provided written informed consent.

Subjects were included in the study if they satisfied the following criteria;

Baseline FEV₁ ≥ 70% of predicted (Fulambarker 2004, Quanjer 1983).

Physician diagnosed asthma.

Step one of treatment by GINA (2006) guidelines (GINA 2006).

Age 18-75 years.

For female patients: menopausal >2yr or using efficient contraception and having a negative pregnancy test.

Positive skin prick test to house dust mite (HDM) (*Dermatophagoides pteronyssinus*).

PC₂₀ to methacholine of <8mg/ml.

Exclusion criteria for the study are detailed in Appendix 1, subjects clinical characteristics are shown in Appendix 2.

***In vitro* study**

Again, volunteers were recruited by advertisement and using the database of volunteers held by the respiratory department. Protocols for the study were approved by the Southampton and South West Hampshire Research Ethics Committee (approval numbers 08/H0502/6 and 05/Q1702/165), and all subjects provided written informed consent.

Asthmatic volunteers were required to have;

Baseline FEV₁ >40% of predicted (Fulambarker 2004, Quanjer 1983).

Physician diagnosed asthma (>1year duration).

PC₂₀ to methacholine of <8mg/ml.

Current regular anti-asthma medication.

Non asthmatic volunteers were required to have;

No diagnosis or history of respiratory disease.

No significant response to methacholine administered at 16mg/ml.

All volunteers were required to be;

Age 18-75 years.

For female patients: menopausal >2yr or using efficient contraception and having a negative pregnancy test.

Full exclusion criteria were the same as the *in vivo* study and are detailed in Appendix 1.

2.2 Skin prick testing.

Skin prick testing was performed using stock solutions of allergen extract (*Dermatophagoides pteronyssinus*, mixed grasses, birch tree pollen, dog fur, cat dander, *aspergillus fumigatus*) (50000 SBU/ml) as well as positive (histamine) and negative (saline, glycerine and phenol) controls (Diagenics Ltd, Milton Keynes, UK). These were applied intradermally by pricking the skin of the volar aspect of the forearm with a sterile lancet through a bead of allergen extract. The mean wheal diameter was recorded (maximum wheal diameter plus wheal diameter perpendicular to that in mm., divided by 2) 15 minutes later.

2.3 Methacholine challenge (screening)

Technique adapted from Chai *et al* and in accordance with the recommendations of the American Thoracic Society (Chai 1975, Crapo 2000).

The standard protocol for methacholine challenge involved an initial assessment of FEV₁ and FVC; providing FEV₁ ≥ 70% and FVC ≥ 80% of predicted the subject continued the challenge, these values were recorded as baseline. The initial challenge step involved 5 breaths of 0.9% saline from an air

driven nebuliser. FEV₁ was recorded 3 times at 1 and 3 minutes after saline inhalation and the best reading from each time point used for analysis. Spirometry was measured using a dry wedge spirometer (Vitalograph, Buckingham, UK).

Doubling concentrations of methacholine (Provocholine, Methapharm Inc., Ontario, Canada) were then delivered by air driven nebuliser, starting with 0.03 mg/ml, to a maximum of 8 mg/ml. FEV₁ was recorded at 1 and 3 minutes following methacholine inhalation. The challenge was stopped when a fall of $\geq 20\%$ from post saline reference was reached. The concentration causing the 20% fall was recorded as a PC₂₀.

2.4 Allergen challenge.

The allergen challenge protocol was adapted from Talyor *et al* (Taylor 2000). Fresh solutions of freeze dried house dust mite (*Dermatophagoides pteronyssinus*) allergen extract (Diagenics Ltd, Milton Keynes, UK) were made up with 0.9% saline to give a working concentration of 5000 U/ml. The solution was administered using a breath activated dosimeter connected to a controlling computer (APS pro nebuliser and controller software (JLAB v. 5.02.0) Viasys Healthcare, Germany). This dosimeter enables delivery of set inhaled doses based on a known concentration of allergen, inhalation time and number of breaths.

Allergen challenge involved an initial assessment of FEV₁ and FVC; providing FEV₁ $\geq 70\%$ predicted and FVC $\geq 80\%$, the subject continued the challenge, these values were recorded as baseline. The initial challenge step involved 5 breaths of 0.9% saline delivering a total dose of 0.1 mg. FEV₁ was recorded 3 times at 5 and 10 minutes after saline inhalation and the best reading from each time point used for analysis. The lower of the best readings at 5 or 10 minutes was then used as the reference (post saline) value for further analysis. Unless

a fall in FEV₁ of >10% from baseline following saline occurred, the subject then inhaled incremental doses of allergen.

Initial dosing of allergen was with 5 U. FEV₁ was then recorded at 5, 10 and 15 minutes, as above, the lowest of these values taken for analysis. Allergen was then administered at 10, 40, 160, 720, 2100 and 5000 U with spirometry as above until a fall in FEV₁ of $\geq 15\%$ compared to post saline reference was obtained. If FEV₁ fell by between 10 and 15% compared to post saline reference, the next dose to be delivered was halved.

FEV₁ was then measured at 20, 30, 45 and 60 minutes; then at 30 minute intervals until 10 hours following the final allergen dose.

On subsequent allergen challenges, initial saline challenge was followed by allergen challenge. The total dose of allergen that had caused a $\geq 15\%$ fall in FEV₁ on the previous occasion was calculated and half of this dose delivered as a bolus, with FEV₁ measurements as above. If a fall of $\geq 15\%$ occurred, the challenge was terminated, if not a quarter of the previous total dose was administered and FEV₁ again measured. If a fall of FEV₁ $\geq 15\%$ did not occur, another quarter of the previous final dose was administered, to give a cumulative dose the same as the previous dose causing a $\geq 15\%$ fall in FEV₁. If a fall of 15% had not occurred, the dosing regime for the initial challenge was then followed. Following an FEV₁ fall of $\geq 15\%$, FEV₁ measurements were taken as above (Grainge 2010).

2.5 Methacholine challenge (repeated)

Repeated methacholine challenges were performed as the screening challenge except that the target drop in FEV₁ was $\geq 15\%$ and the dose calculated to achieve this fall from the screening challenge was administered as a bolus.

FEV₁ was measured at 20, 30, 45 and 60 minutes; then at 30 minute intervals until 10 hours following the final methacholine dose.

2.6 Saline challenge

Following an initial assessment of FEV₁ and FVC as above, the initial challenge step involved 5 breaths of 0.9% saline from an air driven nebuliser. FEV₁ was recorded 3 times at 1 and 3 minutes after saline inhalation and the best reading from each time point used for analysis. Spirometry was measured using a dry wedge spirometer (Vitalograph, Buckingham, UK).

Normal saline was then delivered by air driven nebuliser for 5 breaths taken from functional residual capacity (FRC) to total lung capacity (TLC). FEV₁ was recorded at 1 and 3 minutes following saline inhalation. Following this inhalation, FEV₁ was then measured at 20, 30, 45 and 60 minutes; then at 30 minute intervals until 10 hours following the saline challenge.

2.7 Salbutamol and methacholine challenges.

In order to attempt to differentiate between responses due to the direct chemical action of methacholine and any caused by the airway response to bronchoconstriction, a salbutamol then methacholine challenge protocol was developed. Following an initial assessment of FEV₁ and FVC as above, the initial step was administration via air driven nebuliser of 2.5mg salbutamol. FEV₁ was recorded 3 times at 1 and 3 minutes after the termination of the nebuliser, and the best reading from each time point used for analysis. Methacholine was then administered at a concentration twice that which had caused a 20% reduction in FEV₁ at the screening visit by air driven nebuliser over 5 breaths from FRC to TLC. FEV₁ was again measured 1 and 3 minutes following the administration of methacholine, and at 20, 30, 45 and 60 minutes; then at 30 minute intervals up to 10 hours.

2.8 Symptom scores

Subjects recorded symptom scores and use of reliever medication daily in the week preceding and the week of repeated challenges. The diary cards used are shown in Appendix 3. Minimum daily symptom score was zero, maximum 30.

2.9 Bronchoscopy

Fibreoptic bronchoscopy was performed according to British Thoracic Society (BTS) guidelines (BTS Bronchoscopy Guidelines Committee, 2001) and the local departmental standard operating procedure in the Wellcome Trust Clinical Research Facility. Samples were randomly taken from either the right or the left lung at the initial bronchoscopy, and the opposite side at the second bronchoscopy.

2.9.1 Bronchial biopsies

Biopsies were taken with disposable alligator forceps (Bard, Ref 100503, size: 1.8mm) (KeyMed (Medical & Industrial Equipment) Ltd., OLYMPUS Group Company, Southend-on-Sea, UK) after application of local anaesthetic from carinae of the upper, middle / lingula and lower lobes (6 – 8 biopsies) and further processed for glycol methacrylate (GMA) embedding and immunohistochemistry, and RNA extraction.

2.9.2 Bronchial brushings

To obtain epithelial cells for tissue culture, a sheathed cytology brush (Olympus BC-202D, Keymed, UK) was rubbed gently against the epithelium of a second or third generation bronchus four to five times, then removed from the bronchoscope. The brush was then agitated in phosphate buffered saline (PBS) to remove the cells. This was repeated six times. Cells were then cultured as described below.

2.9.3 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed by installing 100ml (5 x 20ml aliquots) pre-warmed (37°C) normal saline into the segments of the upper lobes and then recollecting the fluid by suction (approximately 40 – 60ml) into a bronchial lavage fluid trap.

2.9.4 BAL processing

On removal from the subject, BAL fluid was filtered using a 100µm nylon filter (BD Falcon cell strainer, Marathon Lab. Supplies. London, UK) and then centrifuged at 1300G for 10 mins at 4°C. The supernatant was removed and aliquoted prior to storage at -80°C for later analysis. The cells were resuspended in PBS and cytocentrifuge slides (Thermo Shandon Ltd, Runcorn, UK) prepared and stored at -80°C for later analysis.

2.9.5 BAL cell counts

BAL total cell counts were performed using a Neubauer hemocytometer and the trypan blue exclusion method. Differential cell counts were performed manually on cytocentrifuge slides stained with rapid Romanowsky stain (Raymond Lamb Ltd, Eastbourne, UK).

2.10 Processing of tissue into glycol methacrylate

Method adapted from Britten *et al* 1993 (Britten 1993). On removal of biopsy from the subject, the biopsy was initially rapidly assessed for adequate size, and then placed immediately into ice cold acetone (Fisher Scientific, Loughborough, UK) containing 2mM phenyl methyl sulphonyl fluoride (Sigma, Poole, UK) and 20mM iodoacetamide (Sigma, Poole, UK) and fixed overnight at -20°C.

The fixative was replaced with dry acetone (as above) at room temperature for 15 minutes, then replaced with methyl benzoate (Fisher Scientific, Loughborough, UK) at room temperature for a further 15 minutes. The biopsy was then infiltrated with processing solution comprised of 5% methyl benzoate in glycol methacrylate (GMA solution A) (Polysciences Inc., Warrington, USA) at 4°C for 6 hours, the processing solution was changed every 2 hours. The biopsies were then placed in freshly prepared embedding solution comprising 10ml GMA solution A, 70mg benzoyl peroxide (Polysciences Inc., Warrington, USA) and 250µl GMA solution B (Polysciences Inc., Warrington, USA) in Taab flat bottomed capsules (Taab, Aldermaston, UK cat no 0094) at 4°C for 48 hours. Biopsies were then stored until required in an airtight box containing silica gel at -20°C.

2.11 Sectioning of GMA embedded tissues

Following rough trimming, GMA embedded biopsies were cut using a Leica Jung supercut 2065 glass knife microtome (Leica, Milton Keynes, UK) with section thickness 2µm, floated on reverse osmosis water (ROW) containing 1% ammonia, and then transferred to 0.01% poly-L-lysine (PLL) (Sigma-aldrich, Poole, UK) coated glass slides (Knittel Glaser, Baunschweig, Germany) for further processing. Slides were stored at -20°C for up to 2 weeks prior to use.

2.12 Staining procedure for GMA embedded tissue

Biopsy sections on PLL coated slides were initially incubated with 0.1% sodium azide (Fisher Scientific, Loughborough, UK) and 0.3% hydrogen peroxide (Sigma-Aldrich, Poole, UK) in ROW for 30 mins to inhibit endogenous peroxidases. The slides were then washed with tris buffered saline (TBS) for 3 x 5 minutes, prior to the addition of blocking medium (Dulbecco's modified eagles medium (DMEM) with 20% fetal bovine serum (FBS) and bovine serum albumin (BSA)) for 30 minutes. Slides were then drained and primary

antibodies applied at appropriate dilutions (as determined by titration) under coverslips overnight at room temperature.

Slides were then washed with TBS for 3 x 5 mins, drained and biotinylated second stage antibodies applied at appropriate dilutions for 2 hours at room temperature. Slides were washed again with TBS for 3 x 5 mins, drained, and streptavidin biotin-peroxidase complexes (stABC-HRP complex, Dako, Stockport, UK) applied for 2 hours at room temperature. Following TBS wash (3 x 5 mins) either 3-amino, 9-ethylcarbazole (AEC) (AEC substrate pack, Launch diagnostics, Longfield, UK) or diaminobenzidine (DAB) (liquid DAB substrate pack, Launch diagnostics, Longfield, UK) substrates were applied for 20 or 10 minutes respectively at room temperature as required.

Slides were rinsed in TBS and then running water for 5 minutes, prior to counterstaining with Mayer's haematoxylin (90 seconds) and a further running water rinse. Finally sections were sealed with aqueous mounting medium (AbD Serotec, Kidlington, UK) incubated at 80°C for 30 minutes and allowed to cool prior to coverslipping using Pertex (Surgipath, Peterborough, UK). Primary antibodies used in immunohistochemistry, their source, chromogen and working dilution are shown in Table 2-1. All antibody dilutions were established by titration, and absence of non specific staining established by isotype controls. Antibodies which were tested, but could not be used due to insufficient or non specific staining were Phospho-Smad3 (Ser423/425) rabbit monoclonal antibody (clone C25A9) (New England Biolabs (UK)), RELM β mouse monoclonal antibody (Clone 250911) (R&D systems), TGF β 1 mouse monoclonal (clone TB21) (Abcam), TGF β 1,-2,-3 mouse monoclonal antibody (clone 1D11) (R&D systems) and Activin A mouse monoclonal antibody (clone 132815)(R&D systems).

Table 2-1 Primary antibodies used in immunohistochemistry, their source, chromogen and working dilution.

Antigen	Clone / cat no.	Supplier	Type	Chromogen	Dilution
TGF β	ab50716	Abcam	Rabbit poly	DAB	1:200
Tenascin	M0636	Dako	Mouse mono	DAB	1:60
Collagen III	IE7-D7	Chemicon	Mouse mono	DAB	1:5000
Eosinophils	EG2	Diagnostics development	Mouse mono	AEC	1:10000
Macrophages (CD68)	PG-M1	Dako	Mouse mono	AEC	1:200
Mast cells (tryptase)	AA1	Dako	Mouse mono	AEC	1:1600
RELM β	ab84224	Abcam	Rabbit poly	DAB	1:150
TIMP1	4D12	Calbiochem	Mouse mono	DAB	1:60
MMP9	4H3	R&D Systems	Mouse mono	DAB	1:120
OPN	AF1433	R&D Systems	Goat poly	DAB	1:50
Ki67	MIB1	Dako	mouse mono	DAB	1:250

Mono = monoclonal, poly = polyclonal, cat no = catalogue number, DAB = diaminobenzidine, AEC = 3 - amino, 9 - ethylcarbazole. Abcam, Cambridge, UK. Dako, Ely, UK. Chemicon, Watford, UK. Diagnostisitics Development, Uppsala, Sweden. Calbiochem, Darmstadt, Germany. R&D Systems, Abingdon, UK.

RELM β

No previous immunohistochemistry work had been performed using antibodies to human RELM β , therefore three different antibodies were tried, and optimal concentrations and conditions for staining ascertained. Antibodies tested included rabbit anti human resistin like molecule beta polyclonal antibody R1587-22B (Strattech Scientific Ltd., Newmarket, UK), monoclonal anti human RELM β antibody MAB2917 (R&D systems, Abingdon, UK), and rabbit anti human RELM β polyclonal antibody ab84224 (Abcam, Cambridge, UK). Of the three, the ab84224 polyclonal antibody from Abcam gave the most discrete staining at the lowest titration. RELM β was principally identified in the epithelium, shown at low power with recombinant protein preabsorption control at Figure 2-1 and at high power with isotype antibody control at Figure 2-2.

Figure 2-1 RELM β immunostaining at low power with recombinant protein preabsorption control (lower). Bar = 100 μ m.

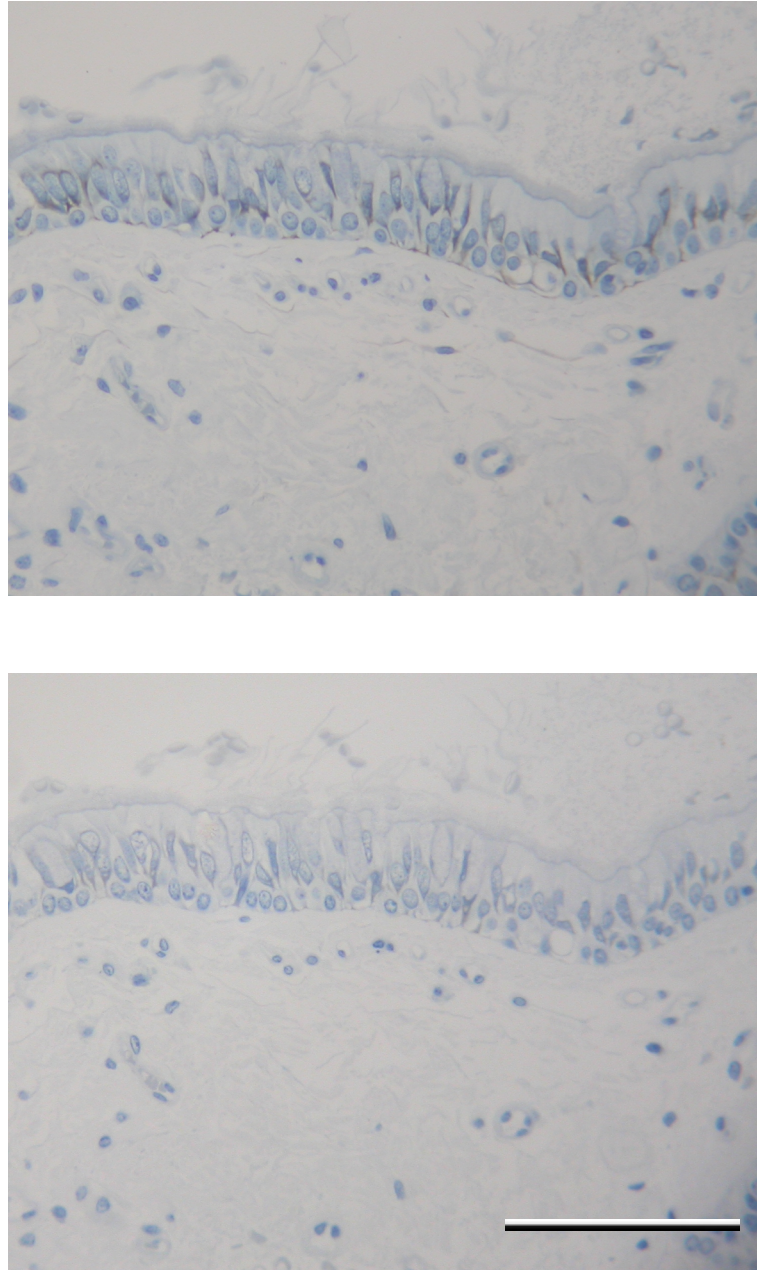
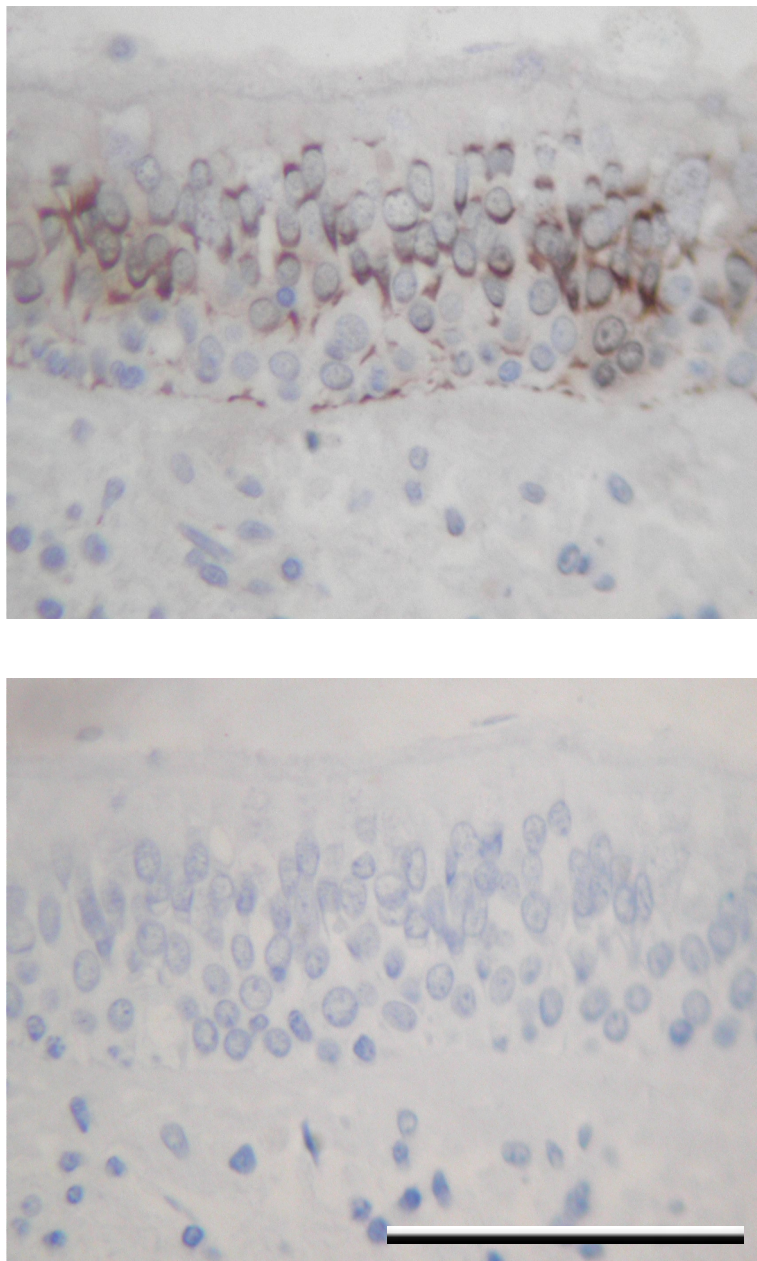


Figure 2-2 RELM β immunostaining at high power with isotype antibody control (lower). Bar = 100 μ m.



2.13 Periodic acid-Schiff staining

Initial experiments were performed to optimise staining with periodic acid-Schiff (PAS) on GMA embedded biopsies. Biopsy sections on PLL coated slides were incubated with periodic acid for 10 minutes at room temperature, then washed well with several changes of ROW. Sections were then incubated with Schiff's reagent (Sigma, Poole, UK) for 20 minutes at room temperature, and then washed in running tap water for 10 minutes. Slides were then counterstained in Meyer's haematoxylin for 30 seconds and washed again in running tap water for 5 minutes. Sections were then coverslipped using Pertex as above.

2.14 Image analysis

Staining was assessed using computer assisted image analysis (Zeiss KS400 image analysis system, Zeiss, Welyn Garden City, UK). Biopsies were examined manually and areas of epithelium and submucosa identified morphologically. The length of the epithelium and the area of the submucosa were calculated using computer assisted image analysis as shown in Figure 2-3 and Figure 2-4. The percentage of the epithelium that stained positive for TGF β and RELM β were calculated by image analysis thresholding of positive staining and exclusion of all areas of the biopsy not of interest, the same method was used to calculate the area of submucosa staining with collagen III as shown at Figure 2-5. This method was not reliable with the colouration of the PAS stain and therefore positive areas of PAS staining were demarcated on enlarged digital images, and the areas calculated as a percentage of the total epithelial area as shown at Figure 2-6. The thickness of the basement membrane and lamina reticularis was calculated beneath epithelium that was cut perpendicular and of full height as shown at Figure 2-7. Cell counts were performed by counting positively stained cells manually; they were then expressed as cells per mm length of epithelium or per square mm of submucosa (the length of epithelium or area of submucosa calculated as above).

A minimum of two biopsy sections separated by at least 30 μ m were examined for each immunohistochemical stain in each patient both before and after inhalation challenge, mean results from this repeated sampling were used for further calculations. Samples from all four exposure groups were intermingled during processing and analysis, which was performed blinded.

2.14.1 Image presentation

Scale bars were added to images as required by photographing a micrometer scale graticule using the same microscope lens, camera and computer acquisition settings as the image in question. Using Photoshop CS5 software (Adobe Systems Inc., San Jose, CA, USA) a bar was overlaid onto the graticule picture, adjusted to an appropriate length, then copied onto the photomicrograph of interest. Any scaling of the image therefore maintains the scale bar in proportion to the image.

Figure 2-3 Method of calculating length of epithelium in bronchial biopsy. Full height perpendicularly cut epithelium is identified morphologically and computer assisted image analysis software used to delineate this (green line). The computer then calculates the length of the epithelium. Bar = 200 μ m.

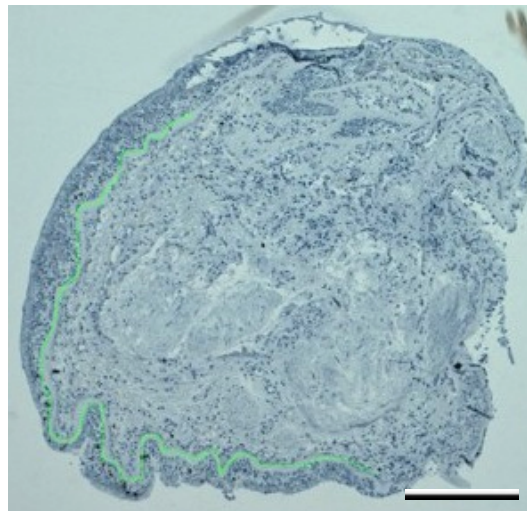


Figure 2-4 Method of calculating biopsy area. A. Photomicrograph of biopsy stained with Meyer's haematoxylin. B. Computer assisted image analysis software is used to delineate the submucosal areas of the biopsy, excluding any areas not of interest, for example muscle and glands which are also identified morphologically. C. Computer analysis calculates area of regions of interest (shown in white). Bar = 200 μ m.

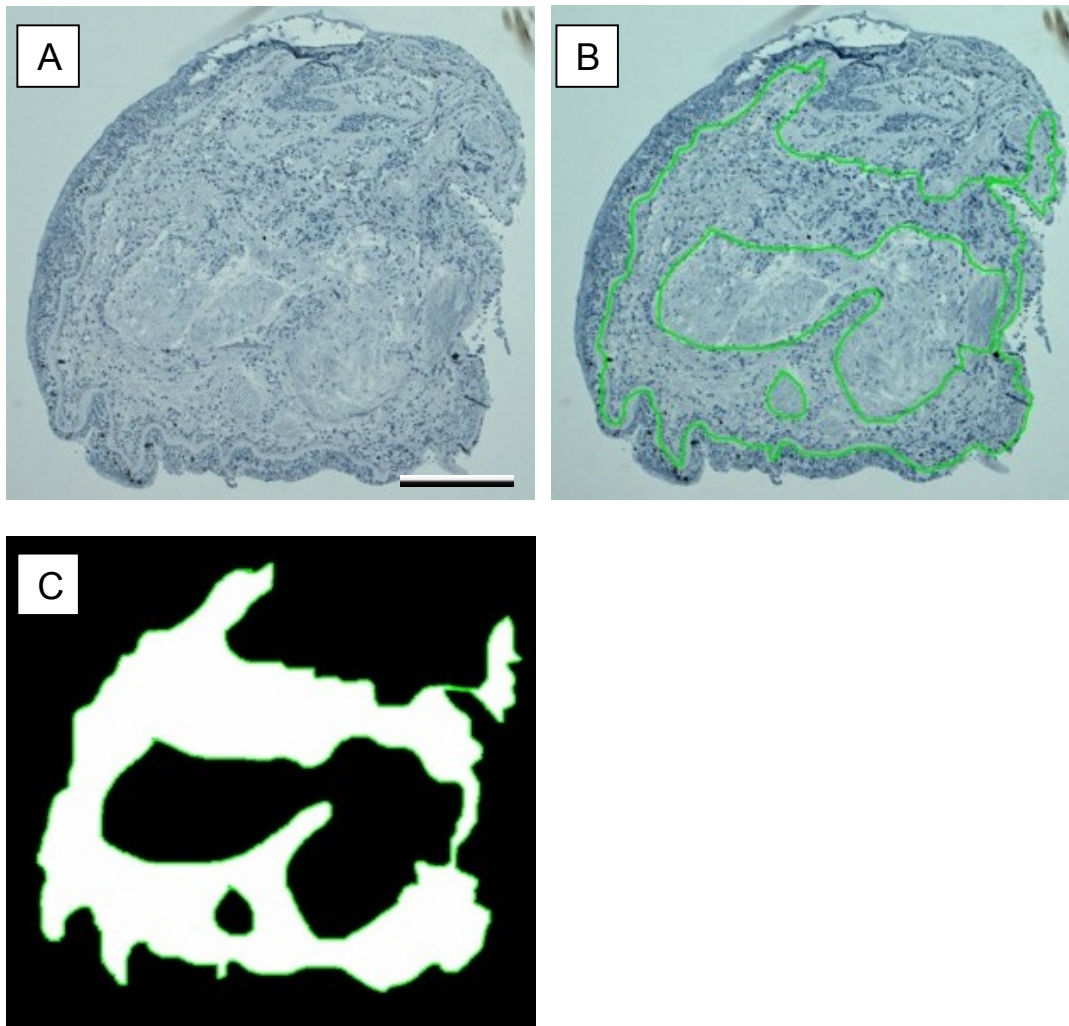


Figure 2-5 Method of calculating submucosal collagen area. A. Photomicrograph of biopsy stained using anti collagen III antibody. B. Computer assisted image analysis software is used to delineate collagen III staining by thresholding the image for brown staining. C. Area of interest in delineated manually to exclude areas of muscle, glands etc. the area of thresholded staining within the area of interest is then calculated as a percentage. Bar = 150 μ m

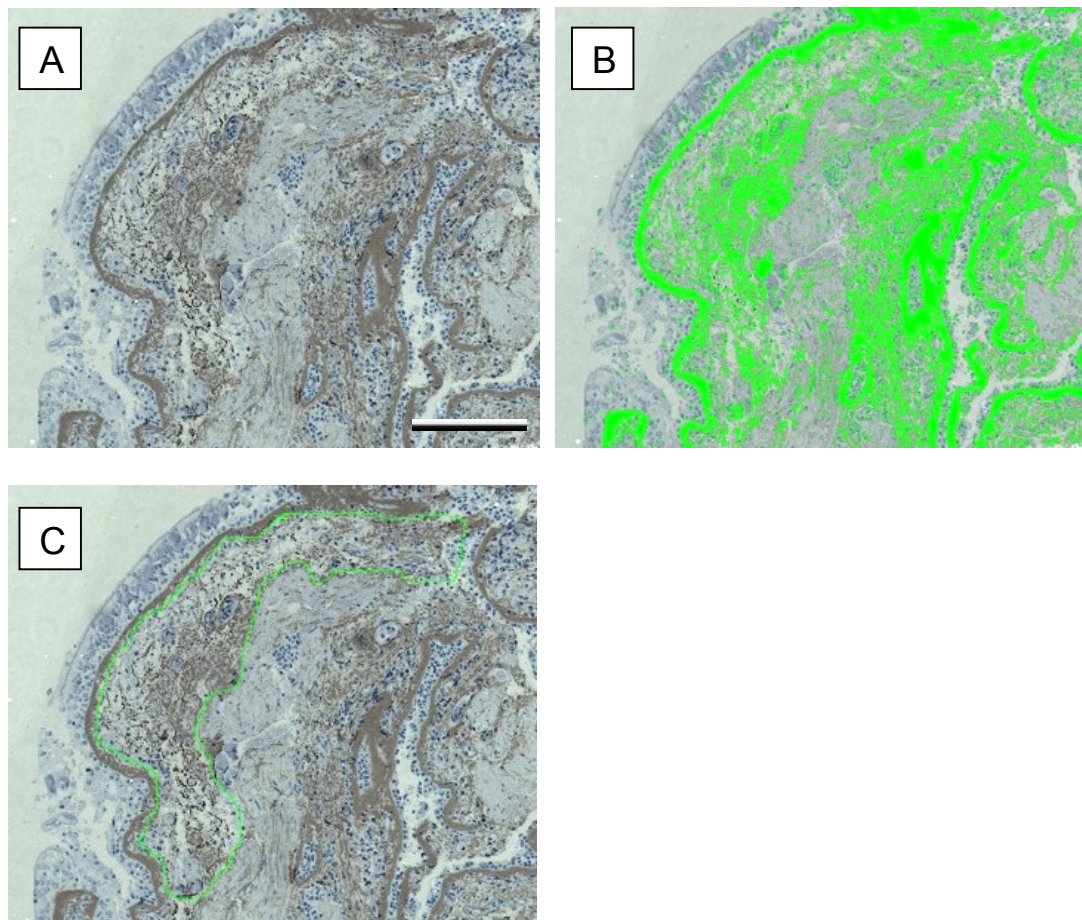


Figure 2-6 Method of calculating percentage of positive periodic acid Schiff staining. A. Area of perpendicularly cut epithelium is identified morphologically in a section stained with periodic acid Schiff stain. B. Computer assisted image analysis software is used to delineate areas of positive staining and area calculated. C. Total area of epithelium delineated and D. total area of epithelium minus area of positive staining calculated. Bar = 50 μ m

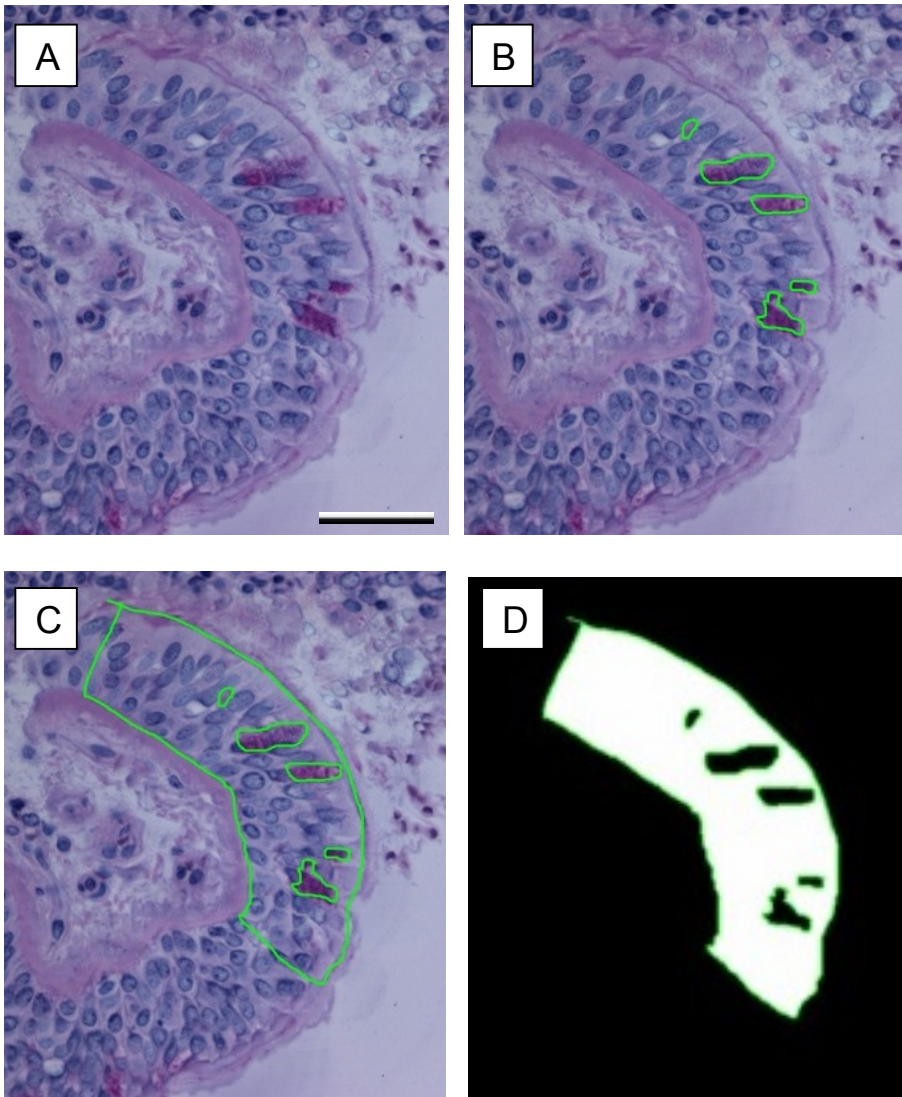
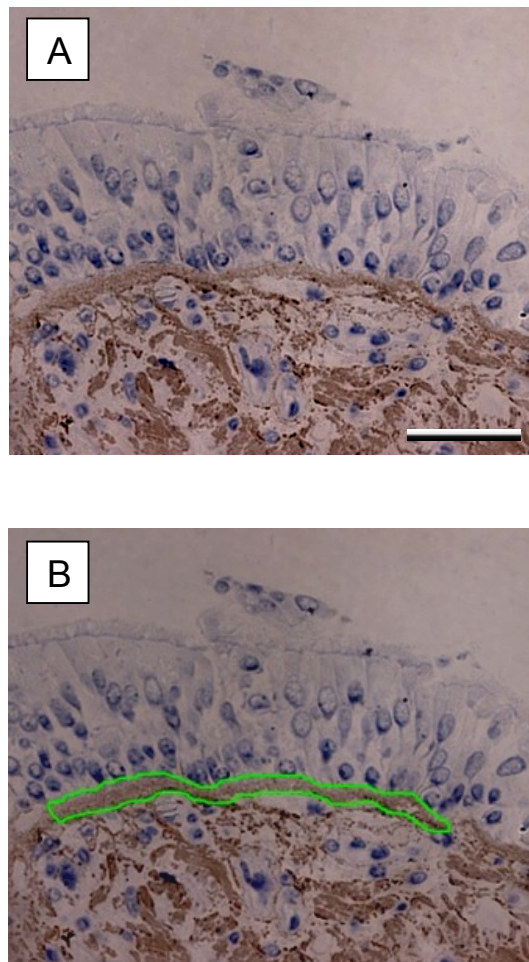


Figure 2-7 Method of delineating collagen band thickness. A. Area of perpendicularly cut epithelium is identified morphologically in a section stained with collagen III antibody. B. Computer assisted image analysis software is used to delineate the collagen band, and this is used to determine its mean thickness. Bar = 50 μ m.



2.15 ELISA

Most ELISA's were performed using commercially available kits according to the manufacturers instructions. The details of these kits are shown in Table 2-2. $\alpha 2$ macroglobulin ELISA was measured using a sandwich ELISA previously developed and validated in the department by Dr Laurie Lau, using capture and detection antibodies from Binding Site (Binding Site, Birmingham, UK) and recombinant $\alpha 2$ macroglobulin from Sigma (Sigma, Poole, UK). Surfactant protein D was measured using an ELISA produced and optimised by members of the research group lead by Professor Tony Postle.

Table 2-2 Commerical ELISA kits used for sample analysis

Target	Manufacturer	Catalogue No.
IL-8	R&D systems	DY208
$\alpha 2$ macroglobulin	N/A	N/A
ECP	MBL (cell biology)	7618E
GroAlpha	R&D systems	DY275
soluble TNFaR2	R&D systems	DY726
Galectin 3	R&D systems	DGAL30
Activin A	R&D systems	DY338
Oseteopontin	R&D systems	DY1433
Surfactant protein D	N/A	N/A
RELM β	AntigeniX America	RHF774CK
TGF $\beta 2$	R&D systems	DY302

R&D Systems, Abingdon, UK. MBL (cell biology), Buckingham, UK. Antigenix America, Huntington Station, NY, USA.

2.15.1 RELM β ELISA

At the start of the project no commercial kits were available for measuring resistin like molecule beta (RELM β), therefore a protocol was developed and optimised. 190 μ l well size multi-sorb ELISA plates (Corning Inc, Nyew York, USA) were coated with 50 μ l of capture antibody (monoclonal mouse anti-human (clone number 250911) RELM β (R&D Systems Europe, Abindon, UK)) in carbonate bicarbonate coating buffer (Sigma Aldrich, Poole, UK) at room temperature overnight. Non specific binding was blocked by incubation with 190 μ l PBS/1% BSA/0.05% Tween 20 for 2 hours at room temperature and the plates washed four times with 190 μ l PBS/0.05% Tween 20. Standard concentrations of recombinant RELM β protein (United States Biologicals, Massachusetts, USA) were added to wells in duplicate in serial doubling dilutions from 10ng/ml to 0.313ng/ml. Samples under investigation were added to wells also in duplicate doubling dilutions. Plates were incubated at room temperature for two hours, and then washed four times, as above. Detection antibody (Biotinylated polyclonal rabbit anti-human RELM β antibody (United States Biologicals, Massachusetts, USA)) was applied to all wells in 50 μ l volume at a concentration of 0.1 μ g/ml for two hours at room temperature. Plates were then washed four times as above. 50 μ l of 0.001% Streptavidin / Horse radish peroxidase in PBS/1% BSA/0.05% Tween 20 was added to each well and incubated for 30 minutes at room temperature. Plates were then washed five times, as above and 50 μ l of 3-3,5,5'-tetramethylbenzidine (TMB) substrate (Invitrogen, Paisley, UK) added to each well for 16 minutes at room temperature. The reaction was terminated with 2M H₂SO₄ and the plate read at 450 and 570nm.

Above concentrations of antibody were determined by performing a sandwich ELISA by the above method using only recombinant protein standard and a variety of capture and detection antibody concentrations until optimum conditions were obtained. Despite system optimisation the sensitivity of the assay was 170 pg/ml when calculated by;

$$\text{Sensitivity} = \left(\frac{\text{background mean}}{\text{low standard mean}} \times \text{low standard mean} \right) + 2 \text{ Standard deviations}$$

Towards the end of the project a commercial kit became available with a detection sensitivity of threshold of 35 pg/ml (Antigenix America, Huntington Station, NY, USA) which was used for the results shown.

2.16 Statistical analysis

Statistical analysis was performed using Prism 3.0 for Windows (Graph Pad Software, San Diego) for area under the curve (AUC) analysis. All other statistical tests were performed using SPSS 17.0 for Mac (SPSS Inc., Chicago). Graphs were prepared using SigmaPlot 10.0 for Windows (Systat Software Inc. California) or Prism 3.0 for Windows. A p value of <0.05 was taken as significant.

The change in values before and after exposure (or the delta) when comparing between the groups is the most valid way of examining changes between four paired samples for various reasons. Firstly it removes any variability between individuals before challenge, by only taking into consideration change in a variable. Also, when compared to multiple within group comparisons, it results in statistical tests being performed once, rather than multiple times, so reducing the risk of a falsely statistically significant result. Finally the between group difference enables any effect of the repeated bronchoscopy, repeated spirometry and any effects of vehicle nebulisation (in the saline group) and direct chemical effects of the methacholine (in the salbutamol group) to be reflected in the statistical testing. Therefore the change or delta calculations are the most valid of the statistical tests, and were performed first. If these are

significant, then the within group differences were also calculated and discussed.

Variables which were normally distributed are presented as mean and standard deviation, and parametric testing performed. Non normally distributed data are presented as median and interquartile range and appropriate non parametric tests performed.

2.17 RNA extraction

2.17.1 Homogenisation of bronchial biopsy tissue

Bronchial biopsies were homogenised in Lysing Matrix D impact-resistant 2.0 ml tubes containing 1.4 mm ceramic spheres (Qiogene, UK) with 1ml of TRIzol® (Invitrogen, Paisley, UK) using a Hybaid RiboLyser Cell Disrupter (Thermo Life Sciences, Hybaid UK) at speed setting 6.0 for 40 seconds. Tubes were cooled on ice for 2 minutes and ribolysed for a second time with the same settings to completely homogenise the bronchial tissue, then cooled on ice for 2 minutes and incubated at room temperature for 5 minutes.

After homogenisation, tissue samples were either processed immediately for RNA extraction or stored at -20°C.

2.17.2 Trizol extraction of cells grown on transwells

250ul of Trizol was added to each transwell, agitated by pipette for 1 minute, then incubated at room temperature for 5 minutes. A second minute of pipette agitation followed, the sample was then pooled with a second transwell into an eppendorf.

2.17.3 RNA extraction (adapted from TRIzol® Reagent protocol)

RNA extractions and RNA processing were performed in a laminar flow hood designated for RNA work. The hood was cleaned with RNaseZap (R-2020, Sigma-Aldrich Company Ltd., Gillingham, UK) and DNA-Remover (Minerva Biolabs GmbH, Berlin, Germany) to prevent RNA degradation and DNA contamination of the samples prior to use. To prevent contamination from pipettes aerosol resistant pipette tips (ART tips, Molecular BioProducts, Inc., San Diego, CA, USA) were used.

200µl of chloroform, minimum 99% (Sigma, UK) per ml of TRIzol was added to tubes containing the sample as has been previously described (Chomczynski 1993). Samples were mixed manually for 15 seconds then incubated at room temperature for 2 to 3 minutes then centrifuged at 12,000g for 15 minutes at 4°C. Centrifugation caused the mixture to separate into a lower red, organic phenol-chloroform phase containing protein, an interphase containing DNA, and a colorless upper aqueous phase, containing RNA. The aqueous phase was transferred into fresh Eppendorf microtubes. 5 to 10µg RNase-free glycogen (Cat. No 10814, Inv, UK) was added to the aqueous phase as a carrier for the RNA.

To precipitate RNA, 500µl of isopropyl alcohol (2-Propanol, for molecular biology, minimum 99%, Sigma, UK) per 1ml of TRIzol Reagent used for the initial homogenisation was added to the aqueous phase. The samples were left to incubate overnight at -20°C to increase the precipitation of RNA. Samples were then vortexed for 10 seconds and then centrifuged at 12,000g for 30 minutes at 4°C. Supernatant was removed and the RNA was washed in 75% ethanol by adding at 1ml of 75% ethanol per 1ml of TRIzol Reagent.

Samples were then mixed and centrifuged at 7,500g for 5 minutes at 8°C. Supernatant was poured off under visual control and the samples pulse spun to

collect all ethanol left on the walls of the tubes, after which the ethanol was carefully removed using a 200µl pipette tip. The pellet was air dried for 5 to 10 minutes before undergoing DNase treatment.

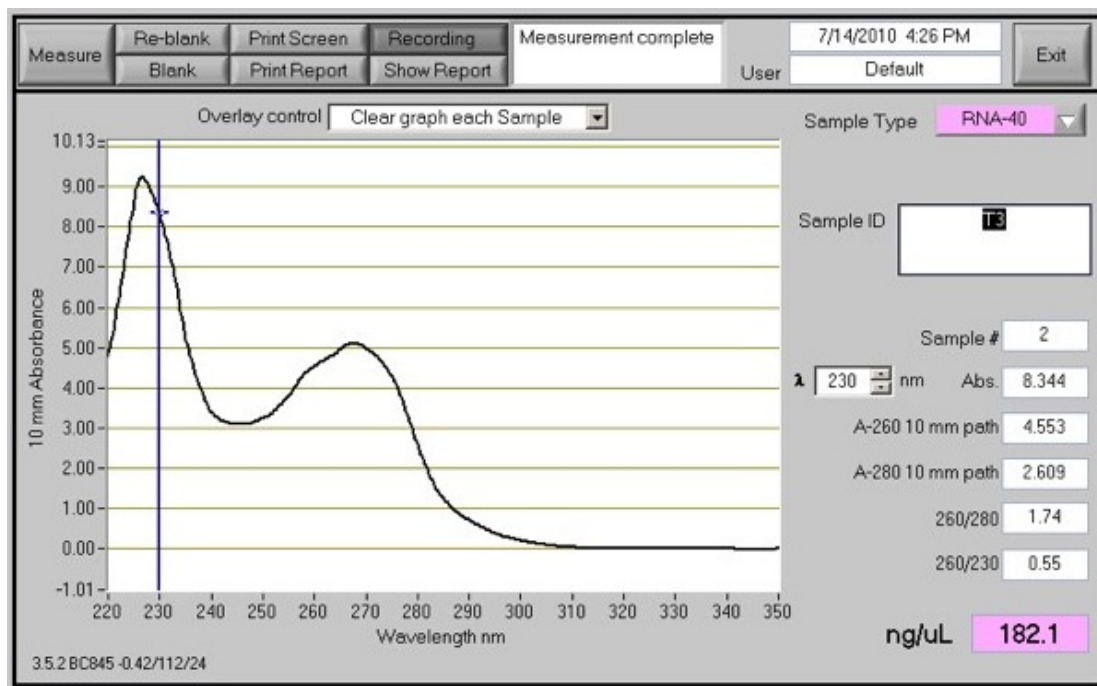
2.18 DNase treatment

To remove contamination by genomic DNA, samples were treated with DNase using DNA-free™ (Cat# AM1906, Ambion, UK) for DNase treatment and removal. The RNA pellet was dissolved in 20µl of total DNase digestion reagents made up of 17µl nuclease-free water, 2µl 10x DNase I buffer (100mM Tris-HCl pH 7.5, 25mM MgCl₂, 5mM CaCl₂) and 1µl of recombinant DNase I (2Units/µl). Samples were incubated in a water bath (Grant W28, Grant Instruments Cambridge, UK) at 37°C for 60 minutes after which the DNase was inactivated by adding 5µl of the DNase inactivation reagent to the samples and incubating them for 2 minutes at room temperature and occasionally mixing them. Samples were then spun at 12,000g for 2 minutes and the RNA stored at -80°C, or immediately used for quantification and reverse transcription.

2.19 Quality check and quantitation of RNA

The quality and quantity of RNA was measured using a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The absorbance at 260 and 280nm was measured and this was used to determine the RNA concentration and purity (pure RNA: A₂₆₀/A₂₈₀ ratio: 1.8 to 2.1) as shown at Figure 2-8.

Figure 2-8 Result from nanodrop ND 1000 spectrophotometer. Total concentration of RNA is shown in ng/ul and a measure of quality of the RNA shown using the 260/280 wavelength absorption ratio.



2.20 Reverse Transcription

The concentration of RNA following RNA extraction from bronchial tissue was measured using Nanodrop (as above) and the volume required calculated to provide 1µg of RNA into the annealing reaction.

2.20.1 Annealing step

1µg of RNA was placed into thin wall tubes with 2µl of oligo dT primer / dNTP mix from Precision Reverse Transcription kit (Primer Design, Southampton, UK). RNA / DNase free water was added to give a final volume of 10µl. The reaction mixture was heated to 65°C for 5 minutes, then snap cooled on ice.

2.20.2 Extension step

An extension mixture comprising 4µl 5X RT buffer, 0.8µl of MMLV RT enzyme (both Primer Design, Southampton, UK), and 5.2µl of RNA / DNase free water was added to the result of the annealing step. The reaction mixture was incubated at 37°C for 15 minutes, then at 42°C for 60 minutes. The resultant cDNA was stored at -20°C until required.

2.21 Quantitative real time polymerase chain reaction (RT qPCR)

2.21.1 Theory of RT qPCR

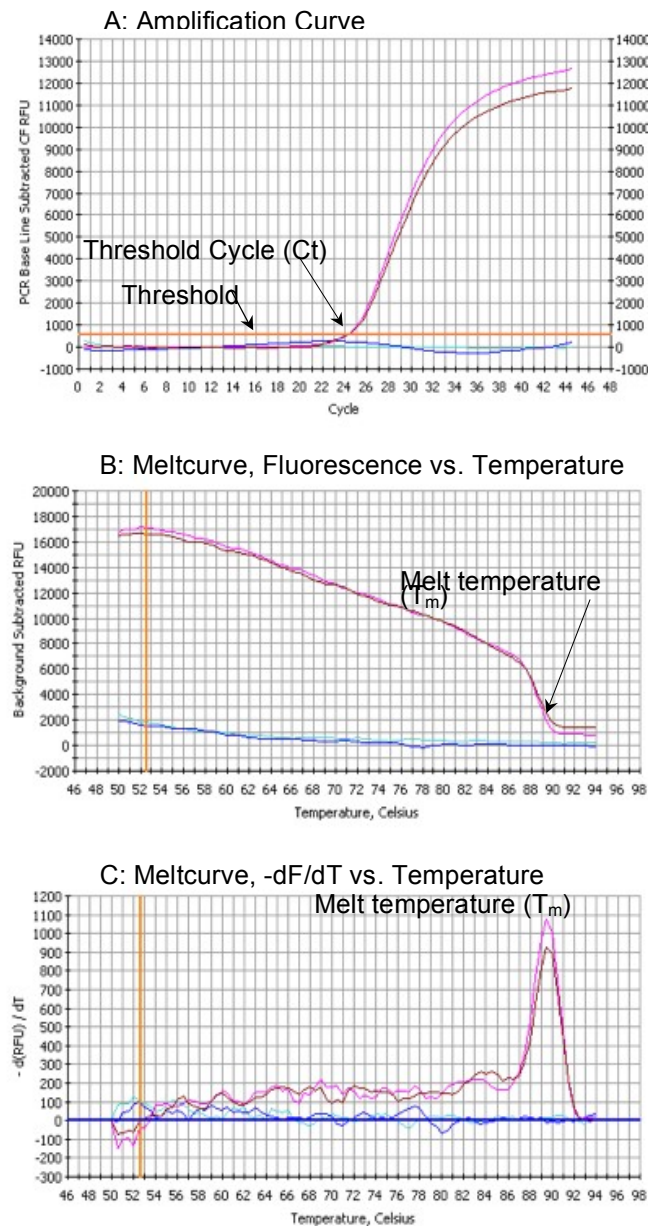
The Polymerase chain reaction (PCR) is an important and widely used tool for amplifying lengths of DNA, it works by a temperature stable polymerase enzyme (Taq polymerase) synthesising a complimentary sequence of bases to any single stranded strand of DNA that has a double stranded starting point. By adding complimentary primers to a sequence of interest in the DNA a target gene may be selected which is then amplified by the polymerase enzyme. Changes in temperature are used to control the activity of the polymerase enzyme, and the binding of the primers to the target gene during the PCR reaction. Initially the temperature is raised to 95°C, denaturing the double stranded DNA into individual strands. The reaction is then cooled to 55°C to allow primer annealing to the regions of interest. This allows polymerase binding and initiation of amplification. The temperature is then raised to 72°C, optimal for polymerase activity. This single cycle of temperature change is then repeated 40 to 50 times to give an exponential amplification of the DNA of interest.

Real time or qPCR is a development of the standard PCR system, by which the amount of DNA product produced by the system can be measured in real time, by the use of fluorescent probes and appropriate detectors. As the number of

copies of the gene of interest is increased, the fluorescence in the reaction increases and can be measured and reported.

Several different systems are available for the measurement of DNA production in real time PCR. Sybr Green is a dye that intercalates into double stranded DNA, only fluorescing when it is intercalated, so detecting the production of double stranded DNA. This system is cheap and effective but has the disadvantage that the dye binding to DNA is not specific, and if areas of DNA are inappropriately amplified, this will be reported as increased fluorescence. Such problems can occur with primer to primer binding, or binding of the primers to genomic DNA contamination in cDNA constructed from RNA samples. In order to overcome this, Sybr primers are designed as exon spanning (to prevent genomic DNA amplification), are used with reverse transcription negative controls, and the products are melted at the end of the amplification to give an indication of the sizes of DNA product produced, with different size products melting at different temperatures, ideally only one such product would be produced, and hence the melt curve would demonstrate only a single peak (Figure 2-9).

In order to give more specificity to the reaction, fluorescent probes can be constructed (such as TaqMan or PerfectProbe) which are around 20-25 bases in length, which contain both a fluorescent reporter dye (eg 6-carboxyfluorescein (FAM)) and a fluorescence quencher at the opposite end (eg. 6-carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA)). When in close proximity, such as when bound to the oligonucleotide probe, the TAMRA prevents the fluorescent emission from FAM being detected. During DNA amplification, the oligonucleotide probe specifically binds to the area of DNA of interest, between the forward and reverse priming sites. During the amplification cycle the polymerase enzyme, which has 3' exonuclease activity, cleaves the FAM from the probe. This allows dissociation of the FAM from the TAMRA quencher, and the presence of fluorescence.

Figure 2-9 Amplification and melt curves of Sybr Green RT qPCR

A: Typical amplification curve generated with iCycler iQ software (BIO-RAD, Hercules, CA, USA) for Collagen I Sybr Green gene detection assay (similar results are obtained from TaqMan and PerfectProbe assays). It shows the sample amplification curve (purple) crossing the threshold set at 550 at the threshold cycle (Ct) at 24.4 and the control sample (RT- control) (blue) with no amplification. B: Meltcurve with fluorescence vs temperature plot showing the steep loss in fluorescence when the melting temperature (T_m) is reached (arrow). C: Meltcurve with negative first derivative of the data shown in B ($-dF/dT$ vs Temperature), showing the specific peak at T_m for the Collagen primers, compared to the absence of melt product with the RT- control.

Method

For real time quantitative PCR both SYBR green (Zipper 2004), TaqMan and Perfect Probe PCR assays were used from Primer Design (Primer Design, Southampton, UK) according to the manufacturers instructions.

Briefly, lyophilised primer mix was reconstituted in RNase/DNase free water, 1µl of this solution was then added to 10µl of 2x Precision Mastermix (containing 0.025U/µl Taq Polymerase, 5 mM MgCl₂, dNTP mix (200 µM each dNTP)) and 4µl of RNase/DNase free water to give a final volume of 15µl. 15µl of this reagent mixture was added to each well of a Thermo-Fast 96 well non skirted PCR Plate (ABgene, Epsom, UK). 5 µl of cDNA of each sample (at concentration of 5ng/ µl) was added to each sample well. Control wells without cDNA but with RNase/DNase free water, or the equivalent concentration of RNA were included on each plate. Amplification was performed according to Primer Design recommendations as shown in Table 2-3 and Table 2-4. The iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hertfordshire, UK) was used for all samples.

2.22 Genes investigated by rtPCR

The genes investigated by rtPCR were resistin like molecule beta (RELMβ), Collagen III (COL3A1), Collagen I (COL1A1), mucin 5AC (MUC5AC), Forkhead box A2 (FOXA2), Tenascin (TNXB), transforming growth factor beta 1(TGFB1), Osteopontin (SPP1), alpha smooth muscle actin (ASMA), a disintegrin and metalloprotease 33 (ADAM33), endothelin 1 (EDN1), endothelin 2 (EDN2) and connective tissue growth factor (CTGF). The primer sequences are shown at Table 2-5 and the amplification protocols at Table 2-6.

Table 2-3 Amplification protocol for real time PCR using SYBR Green detection chemistry. Adapted from Primer Design recommendations.

SYBR Green	Step	Time (sec)	Temp (°C)
Cycling x 50	Enzyme activation	600	95
	Denaturation	15	95
	Data Collection	60	60

Table 2-4 Amplification protocol for real time PCR using Perfect Probe detection chemistry. Adapted from Primer Design recommendations.

Perfect Probe	Step	Time (sec)	Temp (°C)
Cycling x 50	Enzyme activation	600	95
	Denaturation	15	95
	Data Collection	30	50
	Extension	15	72

Table 2-5 Primer sequences for real-time PCR analysis.

Gene	Sense Primer	Anti-sense primer	Dye
ADAM33	GGCCTCTGCTAAACA AACATAATT	TGTCCATGCTGCCACCAA	FAM
ADAM33	PROBE	CCACCCTTCTGTGACAAGCCAG GCT	
COL3A1	GTCCCGCTGGCATTCTG	CTCTCCTTTGGCACCATTCTTAC	Sybr
COL1A1	AGACAGTGATTGAATACAAACCA	GGAGTTTACAGGAAGCAGACA	Sybr
CTGF	CCCAGACCCAACTATGATTGAG	AGGCGTTGTCATTGGTAACC	Sybr
EDN1	TGAGAATAGATGCCAATGTGCTA	GAACAGTCTTTTCCTTTCTTATGATT	Sybr
END2	GTGTCCTCTCCAGCTTTCC	TCTCAAAGTTCTTAGGGCAAATAA	Sybr
FOXA2	GCAGAGCCCCAACAAATG	CGTTGAAGGAGAGCGAGTG	Sybr
MUC5AC	CAGAGGGGTTGACAGTGAC	GAACCGCATTTGGGCATC	Sybr
RETNLB	GTGGTTCGTGGGATGTTCAG	GGGACCCTGGTTTCATTACTG	Sybr
SPP1	GAGGTGATAGTGTGGTTTATGGA	TGATGTCCTCGTCTGTAGCA	Sybr
TNXB	CCGCATGGATGGACAGACA	TCATTGCCAGCCAGAACT	Sybr
TGFB1	CACTCCCACTCCCTCTCTC	GTCCCCTGTGCCTTGATG	Sybr

Table 2-6 Amplification protocols

Taqman gene detection assay		Time	Temp
Enzyme activation	Hot start	10 min	95°C
Cycling x 50	Denaturation	15 sec	95°C
	Data collection	60 sec	60°C
Sybr Green		Time	Temp
Enzyme activation	Hot Start	10 min	95°C
Cycling x 50	Denaturation	15 sec	95°C
	Data collection	60 sec	60°C
Meltcurve	Denaturation	3 min	95°C
Cycling x 90	Data collection	10 sec	50°C + 0.5°C every 10 sec step

2.23 Analysis of RTqPCR data by $\Delta\Delta C_T$ method

Data processing was performed using the iCycler iQ software versions 3.1 (BIO-RAD, Hercules, Ca. USA) and quantification of PCR products from the real time detection was performed using the $\Delta\Delta C_T$ method using Microsoft Excel 2003 for Mac to perform calculations. The cycle number is determined by dropping a perpendicular line from the exponential amplification curve trace when it crosses the threshold mark. This point is the C_t value. The threshold was manually set at 550 for all experiments. These numbers were based on the software calculated threshold, and then standardised across multiple plates to allow comparison between these plates. The C_t values are then used to analyse gene of interest C_t values relative to the expression of normalising or housekeeping genes (minimum of 2). The ΔC_T value is the result of the gene of interest C_t value (eg. Collagen I) minus the geometric mean of the C_t values of the normalising genes (eg. GAPDH and UBC). A difference between two C_t values of 1 unit (=1 PCR cycle) represents a two fold, and of 2 units, a four fold increase in the expression of the target gene. This method relies on the assumption that the normalising genes are stably expressed in each cell or tissue type examined. To make the data easier to interpret and visualise graphically, an arbitrary point from each data set was chosen from which all gene expression was expressed relative to. This was performed differently for the biopsy specimens and for the cell culture work. In the biopsy specimens, for each gene the arbitrary point chosen for this study was the arithmetic mean of the ΔC_T value for all pre-exposure samples. For the cell culture work, the 4 hour non compressed sample was chosen as the arbitrary point for comparison within a single volunteers cells.

Housekeeping genes were also different between the biopsy and cell culture work. Previous work has shown different house keeping genes to be expressed stably in different cells types (Haitchi 2009). In the cell culture work the cells were epithelial and therefore GAPDH and UBC were chosen. For the biopsy

specimens A2 was added as an additional housekeeping gene to compensate for the presence of non epithelial cells such as fibroblasts being present in the homogenised samples.

This manipulation of the ΔC_T calculation is known as the $\Delta\Delta C_T$ method. Relative gene expression can then be calculated by exponential transformation of the $\Delta\Delta C_T$ values.

ie $2^{-(\Delta C_T - (\text{arbitrary } \Delta C_T))}$

2.24 Cell Culture - Air Liquid Interface cultures

2.24.1 Growing cells at an Air Liquid interface

Cells were obtained at bronchoscopy as previously described. Cells in suspension were then centrifuged at 480g for 8 minutes at room temperature, to form a pellet. The supernatant was discarded and the cell pellet resuspended in 1.5ml of bronchial epithelial growth medium (BEGM) (All cell culture medium obtained from Invitrogen, UK). Cells were then counted by the method described above, and grown in T25 (Fisher Scientific, Loughborough, UK) flasks using BEGM medium changed daily until the cells reached 70-80% confluence. The cells in the T25 flasks were then trypsinised and the contents placed into T75 flasks (Fisher Scientific, Loughborough, UK), and the cells allowed to reach 70% confluence. At this point the cell monolayer was trypsinised from the T75 flask and spun down (as above) and the cells counted. Cells were resuspended at a concentration of 5×10^5 cells/ml in BEGM and 200 μ l of the cell suspension applied to each transwell in a 24 well plate (Corning Life Sciences, Amsterdam, Netherlands). Prior to plating, transwells were coated with 200 μ l of Collagen I diluted 1 in 100 with sterile ROW, incubated for 30 minutes at 37°C, the fluid removed from the transwells and the cells added. Hank's buffered salt

solution (HBSS) (1ml) was added to all unused wells in the plate. Cells were then incubated at 37°C in a CO₂ incubator.

The cells were inspected daily, and once they had formed a confluent monolayer, the BEGM was removed from the apical compartment. Simultaneously the BEGM was removed from the basal compartment and replaced with 1xALI medium (see Appendix 7). The date of this procedure was taken as ALI day 0. The cells were then maintained in ALI culture with daily changes of medium and weekly measurement of transepithelial resistance (TER).

2.24.2 ALI culture minimal medium

16 hours prior to compression ALI medium was removed from the basal compartment of the ALI cultures and replaced with minimal medium, comprising a 1:1 mixture of DMEM and bronchial epithelial basal medium (BEBM) plus insulin at a final concentration of 5.7µg/ml, transferrin at a final concentration of 5µg/ml and penicillin and streptomycin at 100units/ml.

2.25 Cell compression system

Following work from Tschumperlin *et al* (Swartz 2001, Tschumperlin 2001, 2003, 2004 & 2006, Park 2009) a cell compression apparatus was established. The complete system is shown in schematic form in Figure 2-10.

Cylinders of compressed 5%CO₂ in air were obtained from BOC Special Gases (BOC Ltd, UK) and stepped down from cylinder pressure to approximately 1 bar pressure using a 2 stage regulator (C202/BS3, BOC Ltd, UK). Gas was then piped via a 0.22µm millex GP filter micropore filter (Millipore, Carrigtwohill, Ireland) to a low pressure regulator (Regulus 3, Tescom, Germany). This regulator enables precise control of pressure from 0 - 45 cm of water (cmH₂O).

Gas was then piped via a T connector to Magnahelic pressure gauge (Dwyer Instruments, Wycome, UK) and downstream to a three way tap (BD Connecta, Becton, Helsingborg, Sweden) allowing pressure venting as needed (Figure 2-11). All tubing from this point was Sterile luer lock tubing (Starstedt, Numbrecht, Germany) heated to 37°C. Gas was then piped via a humidification chamber (Figure 2-12) (Leica, Germany) to a manifold of sequential 3 way taps (Figure 2-13). From this manifold, gas was piped to 17G stainless steel needles (Scientific laboratory supplies, UK) passed through 7mm solid rubber bungs (Fischer Scientific, UK), and cut off flush with the end of the rubber bungs (Figure 2-14). The rubber bungs were then inserted into transwells to establish a pressure tight seal, and pressurised to 15 or 30 cmH₂O as required (Figure 2-15). Non pressurised transwells had the same bungs as shown in Figure 2-15 inserted, but no pressure was applied. The system was maintained in a custom manufactured Perspex box at 37°C and humidified 5%CO₂ piped at atmospheric pressure to the non pressurised cells (Figure 2-16). The entire apparatus from the humidification chamber downstream was established inside a temperature controlled microscope housing Figure 2-17).

Figure 2-10 Schematic diagram of cell pressurisation system.

Components; 1. Cylinder of 5%CO₂ in air, 2. Step down regulator, 3. Regulus 3 low pressure regulator, 4. Magnahelic pressure guage, 5. Humidification chamber, 6. Manifold, 7. Sites of filters as experimental system initially established. Dashed lines indicated heated tubing.

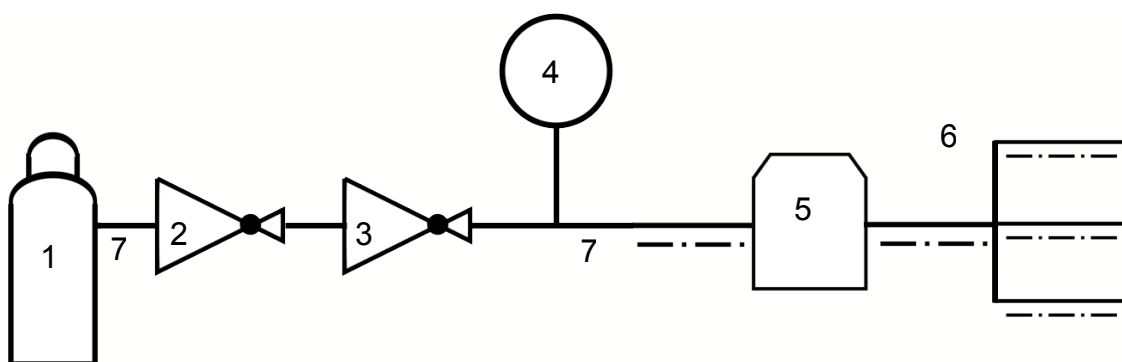


Figure 2-11 Custom manufactured compression apparatus. A. inlet of filtered 5% CO₂ in air at approximately 1bar pressure. B. Low pressure regulator (Regulus 3, Tescom, Germany) enabling precise control of pressure from 0 - 45 cm of water (cmH₂O). C. 'T' connector. D. Magnahelic pressure guage (Dwyer Instruments, Wycome, UK). E. Three way tap (BD Connecta, Becton, Helsingborg, Sweden) allowing pressure venting as needed. F. Micropore filter initially present, then removed (see text).



Figure 2-12 Home made (right) and manufactured (Zeiss, Welwyn Garden City, UK) humidification apparatus.



Figure 2-13 Pressure manifold and bungs.

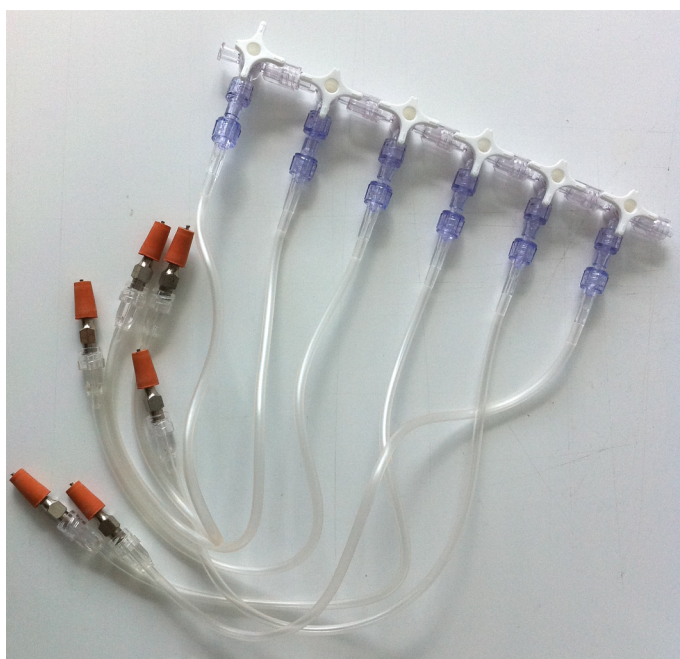


Figure 2-14 Method for sealing and pressurising transwells. 17 Gauge stainless steel needles passed through 7mm solid rubber bungs with the needle end cut off flush with the bung (left). Needle / bung inserted into transwell (right).

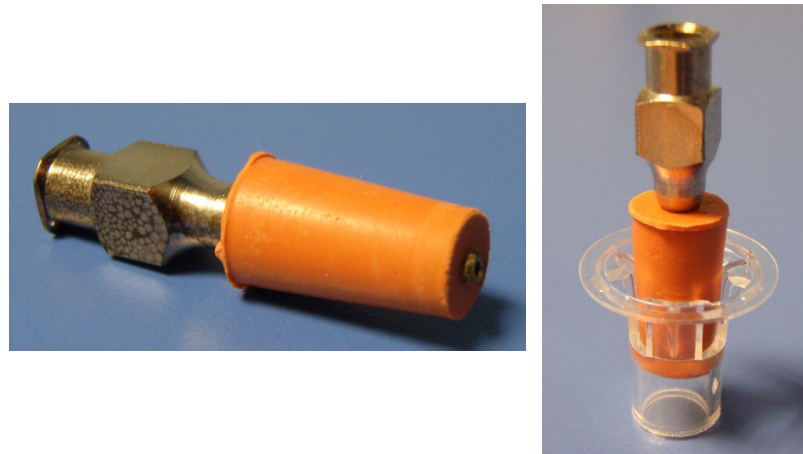


Figure 2-15 Multiple bungs inserted into transwell plate for simultaneous pressurisation of multiple transwells.



Figure 2-16 The cells are contained inside a acrylic enclosure which is filled with 5% CO₂ in air (supplied through the lid via the metal connection on the right) maintained at 37°C. The container is open to the atmosphere to prevent pressurising the control cells.

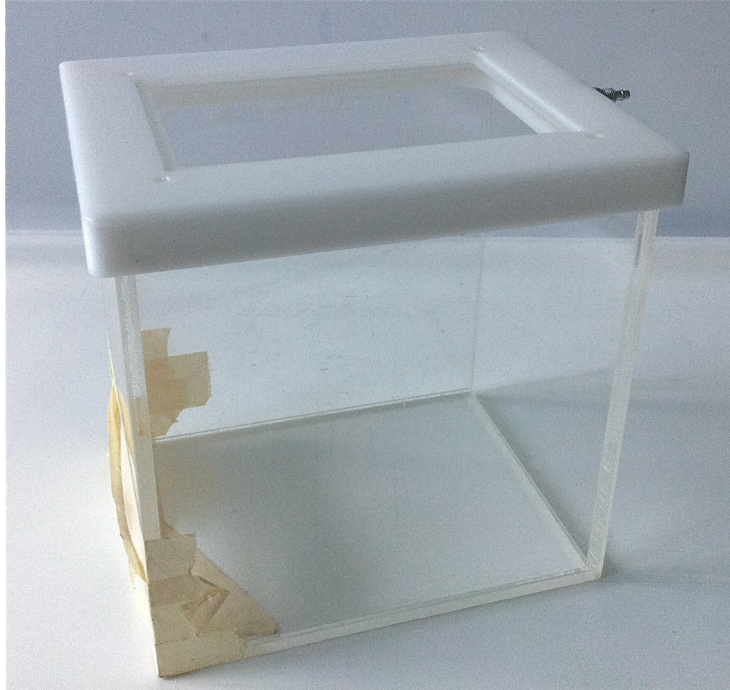
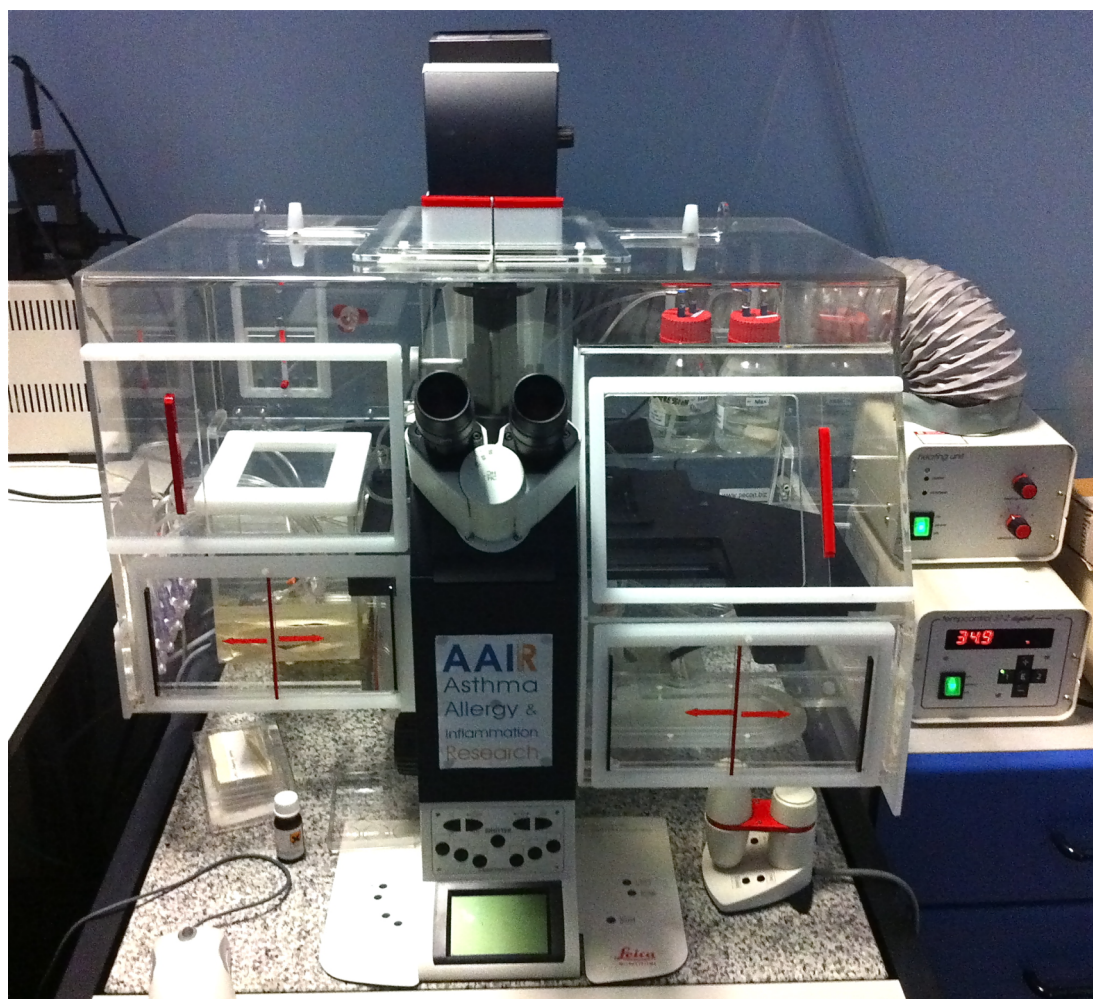


Figure 2-17 The pressurisation apparatus inside a temperature and humidity controlled microscope housing.



2.25.1 System establishment

The compression system was initially set up with a micropore filter immediately distal to the cylinder of compressed gas, and additionally downstream of the pressure regulator, prior to entry to the humidifier and warming box.

2.25.2 Initial experiments

Initially, cells were compressed every other day for 3 compressions to mimic the bronchoconstriction in the human subjects, however several problems were noted with this approach. Previously published work had shown an increase in TGF β and endothelin 24 hours following compression in cells which had been changed to minimal medium 24 hours prior to compression (Tschumperlin, 2003) but when repeated compression had been carried out with normal growth medium present (Park, 2009) no increase in TGF β had been reported. With cells compressed three times, but with normal growth medium, no increase in TGF β production was seen (data not shown).

As a result of these initial experiments, the cells were changed to minimal medium as detailed above and given in the appendices. Compression of the cells following 16 hours of growth in the minimal medium resulted in an increase in total TGF β 24 hours after the compression had finished in the basolateral medium. This protocol was therefore followed for the rest of the experiments.

2.25.3 System optimisation

Initial experiments were completed using the system described above with two micropore filters and with a custom made humidification chamber constructed from a pyrex medium bottle with holes drilled in the screw top, and two needles pushed through, one of which was cut short. This however leaked and a manufactured humidification bottle was bought from Leica (Figure 2-12). Calibration experiments of the new humidification chamber, using a 30cm

column of water, demonstrated that the Leica humidification chamber was insufficiently free flowing and induced a significant pressure drop across it. Therefore the diffuser (a piece of pumice stone) on the inlet pipe was removed, which resulted in very little pressure drop. At the same time the second micropore filter was removed, also after demonstrating a significant fall in pressure across it.

The apparatus was then calibrated using the column of water prior to each use, with the magnahelic pressure gauge being calibrated to provide an accurate pressure read out.

2.26 Cell compression

Established ALI cultures of primary human bronchial epithelial cells were maintained with ALI culture medium (see appendices). The culture medium was changed daily and the TER's measured weekly. 24 hours prior to compression the TER's were measured and culture medium changed as normal. 16 hours prior to compression ALI culture medium was changed to a 'minimal medium' as above.

Cells were then compressed, or subjected to 'sham' compression with bungs inserted into the transwells and atmospheric pressure 5% CO₂ supplied at the pressures and times described in Chapter 6. Cells were then returned to the incubator for 24 hours. After this time, HBSS was applied to the apical surface of the cultures for measurement of the TER, then this wash was kept as an apical sample at -80°C until needed. The basolateral medium was similarly removed and stored. The cells were then lysed in Trizol for RNA analysis.

Chapter 3 Airway challenge study

In order to compare bronchoconstriction in the presence and absence of additional inflammation, and to stimulate the airway multiple times in order to increase the sensitivity of tests for airway remodelling, it was necessary to establish a repeated high dose allergen challenge protocol.

3.1 Background

Human allergen challenge has been performed since 1951, since then human allergen challenge has been performed by various methods. In broad terms these may be considered as 'whole lung' exposure models where allergen is inhaled, and 'partial lung' models where allergen is applied to part of the bronchial tree via a bronchoscope (Julius 2008, Gratziou 1996). Whole lung allergen challenge models typically comprise a single allergen challenge targeted to achieve an FEV₁ drop of between 15 and 30% from baseline and although these challenges are safe and repeatable and correlate well with single environmental exposures, there is debate as to whether single high dose challenges effectively model repeated allergen exposure in the wider environment (Khan 2000, Kariyawasam 2007, Wenzel 2007, Ravensberg 2007, Phipps 2004, Dworski 2001, Taylor 2000, Arvidsson 2007). In order to address this criticism, some groups have developed a repeated low dose allergen challenge model in which there is no target FEV₁ drop, rather a lower dose of allergen (typically 25% of the dose required to trigger an early asthmatic reaction (EAR)) is administered, which may result in an isolated late allergic reaction (LAR). Following such repeated low dose challenges, BHR measured by repeated histamine challenge increases but the repeated low dose challenge results in attenuation of the LAR subsequently triggered by a single high dose challenge (Ihre 1993 and 2006, Palmqvist 2001).

Human exposure to allergen is difficult and potentially hazardous, animal models which intend to emulate asthma often use repeated high dose allergen challenge to provoke responses in the airway and have been used to develop our understanding of the pathology underlying the disease (Tigani 2007, McVicker 2007, Pini 2006, Hirano 2006). Much of the current understanding of the pathology of asthma is based on such repeated allergen challenge animal models, but there is currently no direct human correlate.

Current human allergen challenge protocols, both single high dose and repeated low dose models are useful in understanding the basis of disease, but have insufficiencies which could be addressed by a repeated high dose challenge model. Firstly individuals may be exposed repeatedly to symptomatic doses of allergen during normal activities, and secondly the animal models, which have been so useful in developing our understanding of the disease, have often been developed with repeated allergen challenge; these models need to be validated against human responses in order to support their previous findings and future use.

In addition there is animal and *in vitro* evidence that repeated challenge or repeated mechanical stress may be required to elicit responses in the airway (Zimmerman 2004, Park 2009). It is possible that responses detected only following repeated challenge are actually present following single challenge, but the techniques used for detection may be insensitive, or it may be that the biological systems require repeated stimulation in order to undergo plastic change. If this is the case, single allergen or bronchoconstriction challenges would not detect airway changes which may be occurring outside the laboratory with repeated allergen exposure or bronchoconstriction.

In order to address the hypothesis that repeated allergen challenge with bronchoconstriction and an inflammatory response results in a different airway

reaction to repeated bronchoconstriction in the absence of inflammation, a repeated human allergen challenge model had to be developed.

Previous repeated allergen challenge in humans has been performed, though in small numbers. In 1996 de Bruin-Weller and colleagues (de Bruin-Weller 1996) administered two inhaled challenges with an aim of inducing a 15% fall in FEV₁, whilst another group has performed repeated allergen challenges daily for 4 days using response to ragweed inhalation measured by airways conductance (Rosenthal 1975). In order to give a repeated stimulation, but with a lesser risk of asthma exacerbation in the study group, it was decided that 3 allergen challenges or other airway challenges would be performed at 48 hour intervals, with a bronchoscopy performed at 8 days after the first challenge (4 days after the last), as *in vitro* responses had been detectable by 7 days in previous work (Kariyawasam 2007).

Repeated methacholine challenge was used to induce bronchoconstriction with an expectation that this would not induce an eosinophilic inflammatory response. In order to match the airway responses where possible, the target for the allergen and methacholine challenges was the initial (EAR) response of a fall in FEV₁ of 15% from post saline inhalation baseline.

As repeated bronchoscopy or repeated provocant inhalation or measurement of FEV₁ might induce airway changes, a further control group of repeated saline inhalation challenge was also included, though with no target reduction in FEV₁. Finally in order to attempt to determine if the direct chemical agonist effect of methacholine via cellular M2 and M3 receptors was relevant, rather than the induced bronchoconstriction, a group which underwent salbutamol inhalation then methacholine inhalation was established.

3.2 Aims

To develop, validate and assess safety and acceptability of a repeated high dose allergen challenge model in human volunteers in the presence of suitable controls.

3.3 Methods and Results

Human volunteers were recruited as described in Chapter 2.

3.3.1 Characterisation of volunteers

Following informed consent, subjects medical history was taken, a previous diagnosis of asthma confirmed, and screening tests performed as required. 82 subjects were screened, 24 were excluded (Reasons for exclusion: 16 subjects, PC₂₀ to methacholine >8. 7, skin prick test negative to HDM. 1, BMI > 40). Baseline characteristics of all consented volunteers are shown at Appendix 2. Following screening, 58 subjects entered the study, the timeline for which is shown at Figure 3-1.

A minimum of 14 days after screening, subjects underwent fibreoptic bronchoscopy at which bronchial biopsy and bronchoalveolar lavage samples were taken. In 6 volunteers, bronchoscopy was either not possible or well tolerated; these subjects were excluded from further study, a schematic of the study design and withdrawals throughout is shown at Figure 3-2. Remaining subjects were randomised to one of four groups with replacement of those dropping out of the study, until 12 subjects in each group completed the protocol.

Figure 3-1 Study timeline

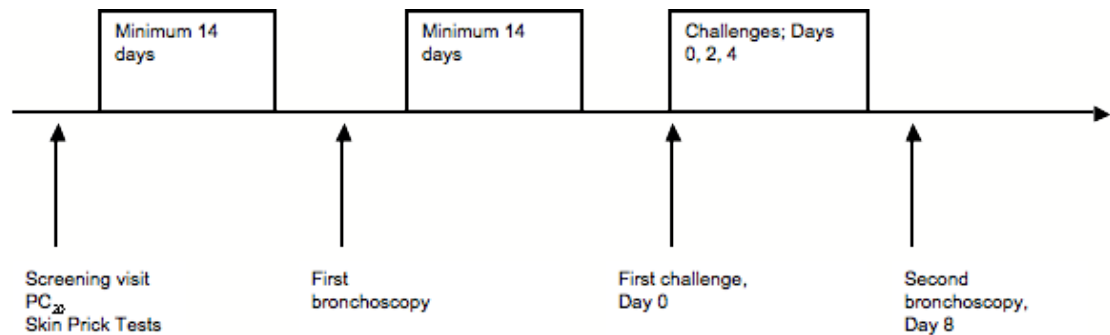
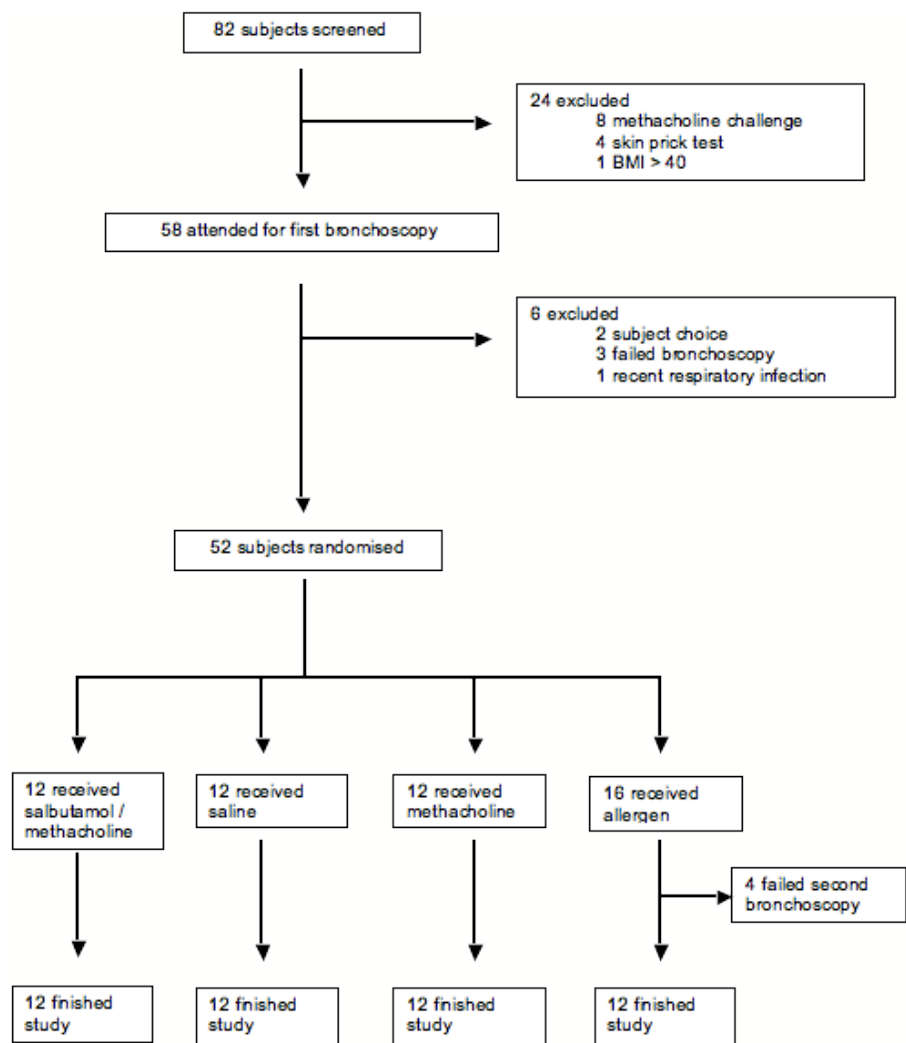


Figure 3-2 Schematic diagram of study design



There were no significant differences in any baseline characteristics between the four subject groups as shown in Table 3-1. Screening FEV₁ and FVC measurements were close to predicted (figures are mean (Standard deviation(SD))) FEV₁; allergen group, 91.1% (11.0), methacholine group, 93.9% (16.3), saline group 93.2% (14.4) and salbutamol group 89.2% (13.5), $p=0.84$ for difference. FVC; allergen 103.5% (10.3), methacholine, 105.5% (16.5), saline, 105.4% (12.4) and salbutamol 108.3% (11.0) $p=0.81$). A PC₂₀ to methacholine of less than 8mg/ml was an inclusion criterion; PC₂₀ values for subjects ranged from 0.06 to 7.93 mg/ml, with median (IQR) of 1.1 (0.5 - 2.2), 1.3 (0.2 - 3.7), 1.7 (1.1 - 2.7) and 2.0 (0.9 - 2.7) for the four groups respectively ($p=0.65$). BMI was similar in all groups; mean (SD) 24.0 (2.2), 23.6 (3.6), 22.8 (2.6) and 22.7 (2.2) $p=0.52$. Skin prick wheal diameters to histamine and house dust mite (HDM) were also similar (Histamine, 4.1 (1.5), 4.2 (1.3), 4.3 (1.3) and 4.7 (1.4) $p=0.73$; HDM, 6.8 (1.8), 6.5 (3.3), 7.4 (3.6) and 7.4 (2.4), $p=0.83$). The ages of the subjects were similar being mean (SD) of 24.0 (4.9), 25.4 (10.4), 21.0 (3.5) and 21.0 (3.5) $p=0.24$. In common with other studies of asthma, there was a preponderance of female volunteers with percentages of female volunteers per group being 75%, 66%, 66% and 83% ($p=0.76$).

The age, BMI, percentage of predicted FEV₁ and FVC, percentage FEV₁ reversibility, skin prick test diameter to histamine and HDM along with PC₂₀ to methacholine are shown for the four subject groups in Figure 3-3, Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7, Figure 3-8 and Figure 3-9.

Table 3-1 Volunteer baseline characteristics

	Allergen (n = 16)	Methacholine (n=12)	Saline (n=12)	Salbutamol (n=12)	p value
Sex (M/F)	4 / 12	4 / 8	4 / 8	2 / 10	0.76
Age (years)	24.0 (4.9)	25.4 (10.4)	21.0 (3.5)	21.0 (3.5)	0.24
% Predicted FEV ₁	91.1 (11.0)	93.9 (16.3)	93.2 (14.4)	89.2 (13.5)	0.84
% Predicted FVC	103.5 (10.3)	105.5 (16.5)	105.4 (12.4)	108.3 (11.0)	0.81
PC ₂₀ methacholine	1.1 (0.5 - 2.2)	1.3 (0.2-3.7)	1.7 (1.1 - 2.7)	2.0 (0.9 - 2.7)	0.65
BMI	24.0 (2.2)	23.6 (3.6)	22.8 (2.6)	22.7 (2.2)	0.52
Skin prick test wheal diameter (Histamine)	4.1 (1.5)	4.2 (1.3)	4.3 (1.3)	4.7 (1.4)	0.73
Skin prick test wheal diameter (HDM)	6.8 (1.8)	6.5 (3.3)	7.4 (3.6)	7.4 (2.4)	0.83

Values are means with SD in parentheses, except for PC₂₀ methacholine which is median (IQR). p values are calculated by comparison of means using ANOVA except for PC₂₀ which uses Kruskal Wallis, and sex, which is calculated by Fishers exact test. HDM - house dust mite, FEV₁ - forced expiratory volume in 1 second, FVC - forced vital capacity, BMI - body mass index.

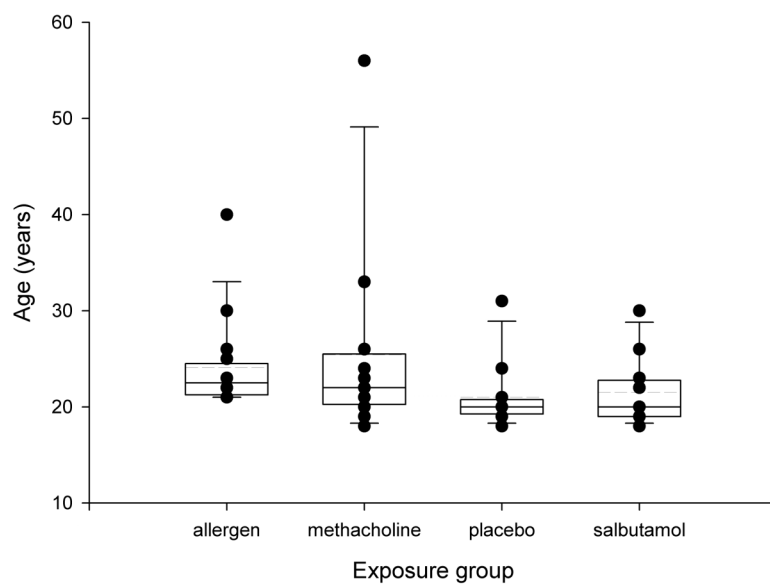
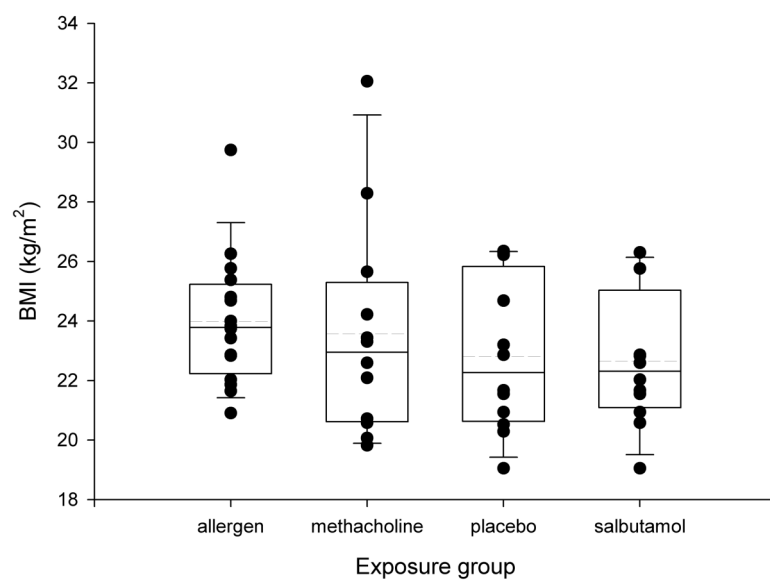
Figure 3-3 Age of volunteers by exposure group.**Figure 3-4 Body Mass Index (BMI) of volunteers by exposure group.**

Figure 3-5 Forced expiratory volume in 1 second of volunteers as percentage of predicted value by exposure group.

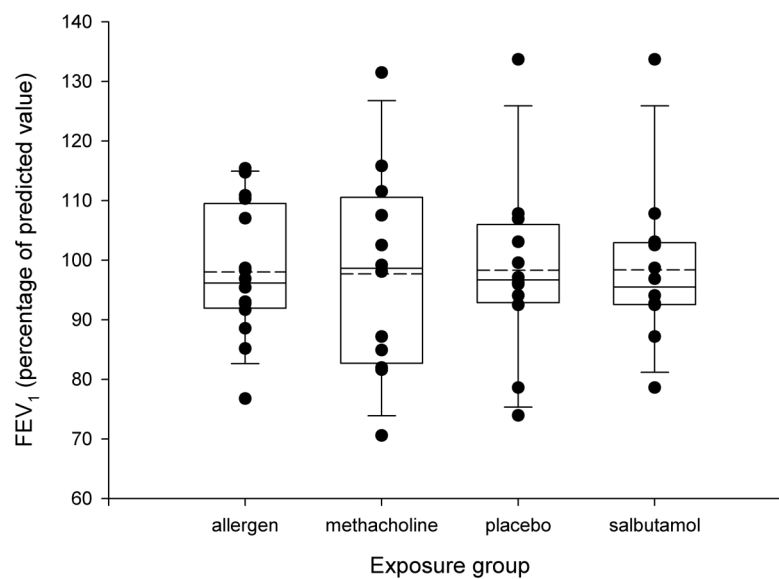


Figure 3-6 Percentage predicted forced vital capacity of volunteers by exposure group.

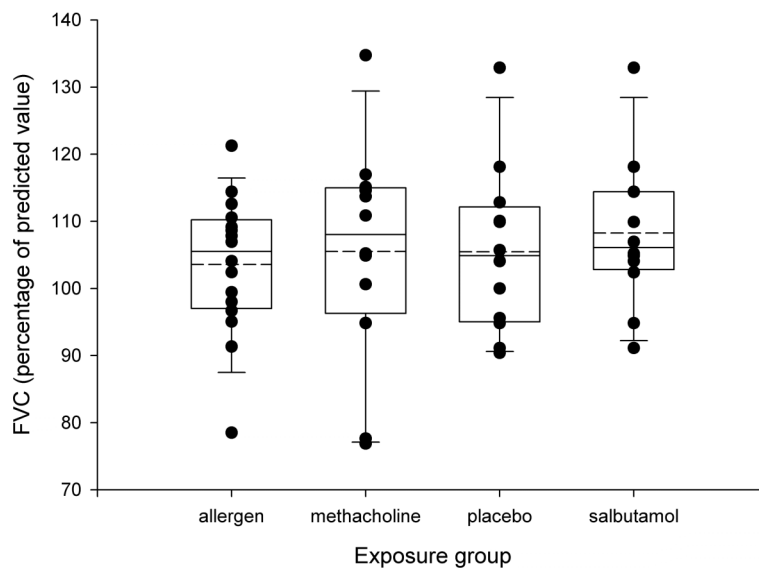


Figure 3-7 Skin prick test wheal diameter in response to histamine in volunteers by exposure group.

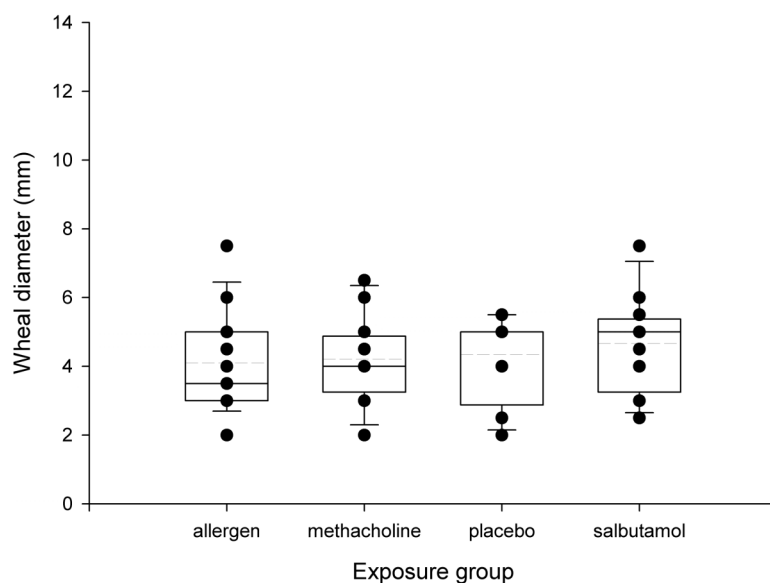


Figure 3-8 Skin prick test wheal diameter in response to house dust mite in volunteers by exposure group.

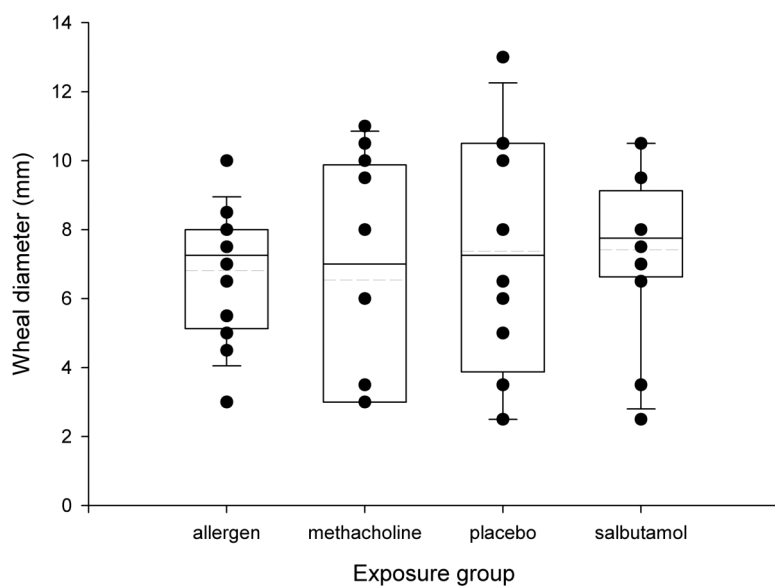
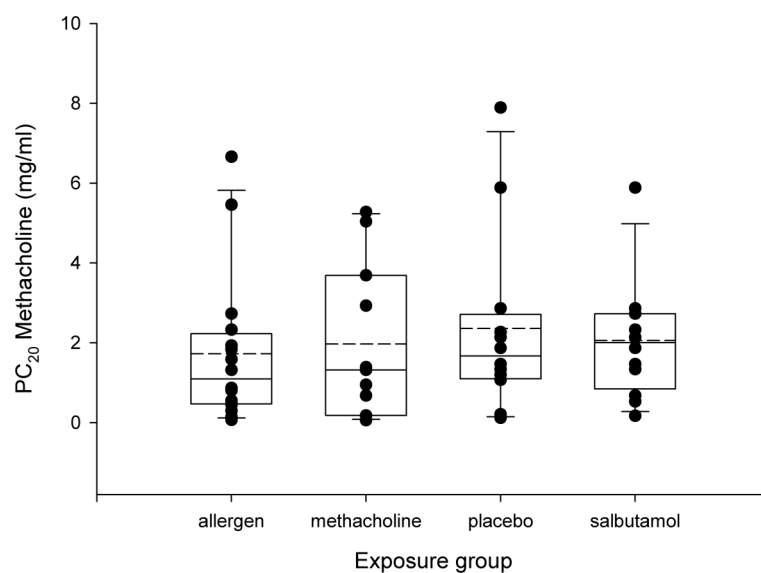


Figure 3-9 Provoking concentration of methacholine required to induce a 20% fall in forced expiratory volume in 1 second in volunteers by exposure group.



3.3.2 Repeated airway challenges

Repeated airway challenges were performed as described in Chapter 2.

3.3.3 Safety

There were no significant adverse events such as worsening of asthma requiring hospital admission or treatment with anti-inflammatory agents.

3.3.4 Magnitude of the early asthmatic reaction

The early asthmatic reaction (EAR) was defined as the fall in FEV₁ from 0-120 minutes following challenge; target fall in FEV₁ was 15% from post saline baseline. EAR following the first challenge resulted in a mean (SD) FEV₁ drop of -20.6% (5.9) in the allergen group, -22.1% (7.5) in the methacholine group, -5.1% (4.7) in the saline group, and in the salbutamol / methacholine group, there was a rise in the FEV₁ from baseline following the salbutamol nebuliser, that was not reversed by methacholine inhalation, mean (SD) rise in FEV₁ in the salbutamol group was 6.9% (7.7). Similar falls were achieved following the second and third challenges (-22.5% (7.0) and -21.7% (8.3) for the allergen group, -23.1% (8.3) and -22.1% (6.9) for the methacholine group, -3.9% (3.0) and -4.9 (2.9) for the saline group and similar rises in the salbutamol and methacholine group of 10.5 (16.1) and 7.5 (8.9). There was no significant within group difference for the EAR change induced during the three challenges (p=0.54 for allergen, 0.89 for methacholine, 0.37 for saline and 0.99 for salbutamol). There was a significant difference between the four groups in terms of EAR change achieved (p<0.001 for all three challenges), however there was no significant difference in EAR between the allergen and methacholine groups for any challenge (p=0.45, 0.98 and 0.95 by Mann-Whitney U test respectively). These data are summarised in Table 3-2 and Table 3-3.

Area under the curve (AUC) was calculated for the EAR for the allergen, methacholine and saline challenges as although the maximal fall in EAR was similar between the allergen and methacholine challenges, the duration of that fall, and hence the area under the curve may have been different. Mean (SD) AUC for the EAR was 1161 (379) in the first allergen challenge, 1174 (590) in the first methacholine challenge and 77 (85) in the initial saline challenge. For the second and third challenges AUC for EAR was 1302 (507) and 1510 (685) for the allergen challenges, 932 (376) and 1320 (671) for the methacholine challenges and 134 (155) and 177 (185) for the saline challenges. AUC was not calculated for the salbutamol challenges as the reason for calculating the AUC was to determine whether the duration of FEV₁ fall was similar in the challenges where such a fall occurred. There were no significant within group differences for EAR AUC, though there was a trend towards greater area in the allergen group (allergen $p=0.07$, methacholine $p=0.13$ and saline $p=0.25$). The first and third allergen and methacholine challenges, in keeping with the mean maximal fall in FEV₁, showed no difference in the AUC (first $p=0.73$, third $p=0.42$ (by Mann-Whitney U) whilst for the second challenge there was a statistically significant difference ($p=0.05$) between the allergen and methacholine challenge groups. These data are summarised in Table 3-4 and Table 3-5.

Across the three challenges, salbutamol nebulisation induced a rise in FEV₁ from mean (SD) percentage predicted of 89.4 (16.5) to 99.0 (13.6) ($p<0.001$). Subsequent methacholine administration did not alter mean FEV₁ as percentage of predicted, following methacholine the mean was 98.9 (13.7) ($p=1.0$). The data are shown at Table 3-6.

Table 3-2 Percentage falls in FEV₁ following allergen, methacholine, saline or salbutamol / methacholine challenges.

	Allergen (n=16)	Methacholine (n=12)	Saline (n=12)	Salbutamol (n=12)	p value
FEV ₁ EAR challenge 1	-20.6 (5.9)	-22.1 (7.5)	-5.1 (4.7)	6.9 (7.7)	<0.001
FEV ₁ EAR challenge 2	-22.5 (7.0)	-23.1 (8.3)	-3.9 (3.0)	10.5 (16.1)	<0.001
FEV ₁ EAR challenge 3	-21.7 (8.3)	-22.1 (6.9)	-4.9 (2.9)	7.5 (8.9)	<0.001
p value within group	0.54	0.89	0.37	0.99	
FEV ₁ LAR challenge 1	-22.8 (13.5)	-8.5 (7.0)	-5.1 (4.7)	-1.4 (5.2)	<0.001
FEV ₁ LAR challenge 2	-24.1 (14.4)	-5.7 (3.8)	-4.5 (4.7)	-0.1 (10.0)	<0.001
FEV ₁ LAR challenge 3	-26.7 (14.4)	-9.7 (8.8)	-5.2 (3.1)	-0.6 (3.6)	<0.001
p value within group	0.68	0.54	0.85	0.77	

Values are means with SD in parentheses. FEV₁ = forced expiratory volume in 1 second, EAR = lowest FEV₁ measured during early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). p values are calculated using Kruskal-Wallis test between groups and Friedman's Test within groups.

Table 3-3 Comparison of change in FEV₁ during early and late asthmatic reaction following repeated inhalation of allergen or methacholine.

	Allergen (n=16)	Methacholine (n=12)	p value
FEV ₁ EAR challenge 1	-20.6 (5.9)	-22.1 (7.5)	0.45
FEV ₁ EAR challenge 2	-22.5 (7.0)	-23.1 (8.3)	0.98
FEV ₁ EAR challenge 3	-21.7 (8.3)	-22.1 (6.9)	0.95
p value within group	0.54	0.89	
FEV ₁ LAR challenge 1	-22.8 (13.5)	-8.5 (7.0)	0.001
FEV ₁ LAR challenge 2	-24.1 (14.4)	-5.7 (3.8)	<0.001
FEV ₁ LAR challenge 3	-26.7 (14.4)	-9.7 (8.8)	<0.001
p value within group	0.68	0.54	

Values are means with SD in parentheses. FEV₁ = forced expiratory volume in 1 second EAR = lowest FEV₁ measured during early asthmatic reaction (0 to 120 mins), LAR = late asthmatic reaction (150 to 600 mins). p values are calculated using Mann-Whitney Test between groups and Friedman's Test within groups.

Table 3-4 Area under the curve analysis of FEV₁ following allergen, methacholine, saline or salbutamol / methacholine challenges.

	Allergen (n=16)	Methacholine (n=12)	Saline (n=12)	p value
AUC EAR challenge 1	1161 (379)	1174 (590)	77 (85)	<0.001
AUC EAR challenge 2	1302 (507)	932 (376)	134 (155)	<0.001
AUC EAR challenge 3	1510 (685)	1320 (671)	177 (185)	<0.001
p value within group	0.07	0.13	0.25	
AUC LAR challenge 1	5455 (3980)	1823 (1924)	575 (497)	<0.001
AUC LAR challenge 2	5881 (3712)	1519 (1230)	657 (787)	<0.001
AUC LAR challenge 3	6645 (4205)	1837 (1039)	743 (700)	<0.001
p value within group	0.47	0.92	0.61	

Values are means with SD in parentheses. EAR = Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). AUC area under the curve. p values are calculated using Kruskal Wallis test (between groups) or Friedmans' test (within groups). For the LAR, comparison between allergen and methacholine groups alone is shown below. Saline compared to allergen p<0.001 for each challenge, saline compared to methacholine p=0.004, 0.04 and 0.007 in the challenges 1,2 and 3 respectively (Mann-Whitney test).

Table 3-5 Comparison of area under the curve for change in FEV₁ during early and late asthmatic reaction following repeated inhalation of allergen or methacholine.

	Allergen (n=16)	Methacholine (n=12)	p value
AUC EAR challenge 1	1161 (379)	1174 (590)	0.73
AUC EAR challenge 2	1302 (507)	932 (376)	0.05
AUC EAR challenge 3	1510 (685)	1320 (671)	0.42
p value within group	0.07	0.13	
AUC LAR challenge 1	5455 (3980)	1823 (1924)	<0.001
AUC LAR challenge 2	5881 (3712)	1519 (1230)	<0.001
AUC LAR challenge 3	6645 (4205)	1837 (1039)	<0.001
p value within group	0.47	0.92	

Values are means with SD in parentheses. EAR = Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). AUC = area under the curve. p values are calculated using Mann-Whitney Test (between groups) or Friedmans' test (within groups).

Table 3-6 Change in percentage predicted FEV₁ at three stages of salbutamol and methacholine challenge; prior to challenge, following salbutamol and following methacholine.

	Challenge number			
	1	2	3	mean
Pre Salbutamol (A)	89.2 (13.5)	89.1 (19.1)	89.8 (17.7)	89.4 (16.5)
Post salbutamol (B)	98.6 (13.1)	99.5 (14.0)	99.0 (14.9)	99.0 (13.6)
p value A/B	0.002	0.002	0.002	<0.001
Post methacholine (C)	98.8 (13.7)	98.8 (14.0)	99.3 (14.5)	98.9 (13.6)
p value B/C	0.88	0.70	0.64	1.0

Mean (SD) percentage predicted FEV₁ before and after salbutamol and after methacholine on 3 occasions and mean of all three. p value calculated by Wilcoxon Signed Ranks Test.

3.3.5 Magnitude of the late asthmatic reaction

The late asthmatic reaction (LAR) was defined as the maximum FEV₁ fall from post saline baseline between 150 and 600 minutes post challenge. This was again assessed by both mean maximal fall during this period, and area under the curve.

Mean lowest FEV₁ recorded during the LAR for the first allergen challenge was -22.8 (13.5), -8.5%(7.0) for methacholine, -5.1 (4.7) for saline and -1.4 (5.2) for salbutamol / methacholine. For subsequent challenges mean LAR was -24.1 (13.5) and -26.7 (14.4) for the allergen challenges, -5.7 (3.8) and -9.7 (8.8) for the methacholine challenges, -4.5 (4.7) and -5.2 (3.1) for saline and -0.1 (10.0) and -0.6 (3.6) for salbutamol / methacholine. There were no significant differences within the groups for maximal mean FEV₁ fall during the LAR ($p=0.68$ for allergen, 0.54 for methacholine, 0.85 for saline and 0.77 for salbutamol) (Table 3-2). There was a highly significant between group difference in all challenges ($p<0.001$), including a difference between allergen and methacholine for each challenge. ($p=0.001$ or less for each challenge). These data are summarised in Table 3-3.

The AUC for the LAR was similarly calculated; Mean (SD) AUC for the LAR was 5455 (3980) in the first allergen challenge and 1823 (1924) in the first methacholine challenge ($p<0.001$). For the second and third challenges AUC for EAR was 5881 (3712) and 6645 (4205) for the allergen challenges and 1519 (1230) and 1837 (1039) for the methacholine challenges ($p<0.001$ for between provocation difference for both challenges (Mann Whitney U test)). There were no within provocation group differences between the three challenges ($p=0.47$ and 0.92 for allergen and methacholine respectively). The AUC for the LAR for the saline challenges was 575 (497), 657 (787) and 743 (700), there was no significant difference within the saline group, though there was a significant difference between the saline and allergen groups ($p<0.001$ for all three

challenges and the saline and methacholine groups ($p=0.004$, 0.039 and 0.007 for the three challenges respectively). These results are summarised in Table 3-4 and Table 3-5.

FEV₁ changes over time, including EAR and LAR following the initial provocative challenge are shown at Figure 3-10, for the second challenge at Figure 3-11 and the final challenge at Figure 3-12. FEV₁ following repeated allergen challenge is shown at Figure 3-13, following repeated methacholine challenge at Figure 3-14, following repeated saline challenge at Figure 3-15 and following repeated salbutamol / methacholine challenge at Figure 3-16. The mean FEV₁ changes following all three challenges are shown in Figure 3-17.

Figure 3-10 Percentage change in FEV₁ over time following first of three allergen, methacholine, saline and salbutamol / methacholine challenges. Mean $\pm$ SEM.

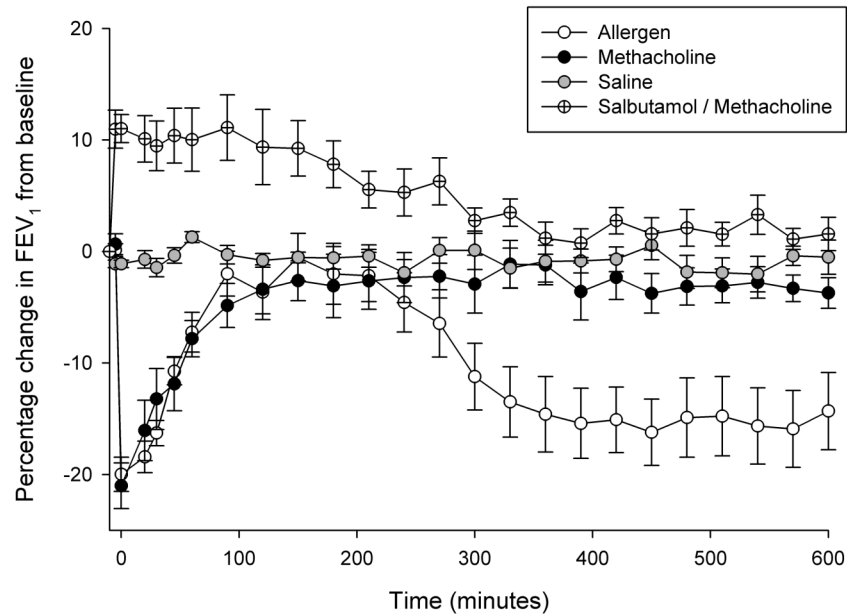


Figure 3-11 Percentage change in FEV₁ over time following second of three allergen, methacholine, saline and salbutamol / methacholine challenges. Mean $\pm$ SEM.

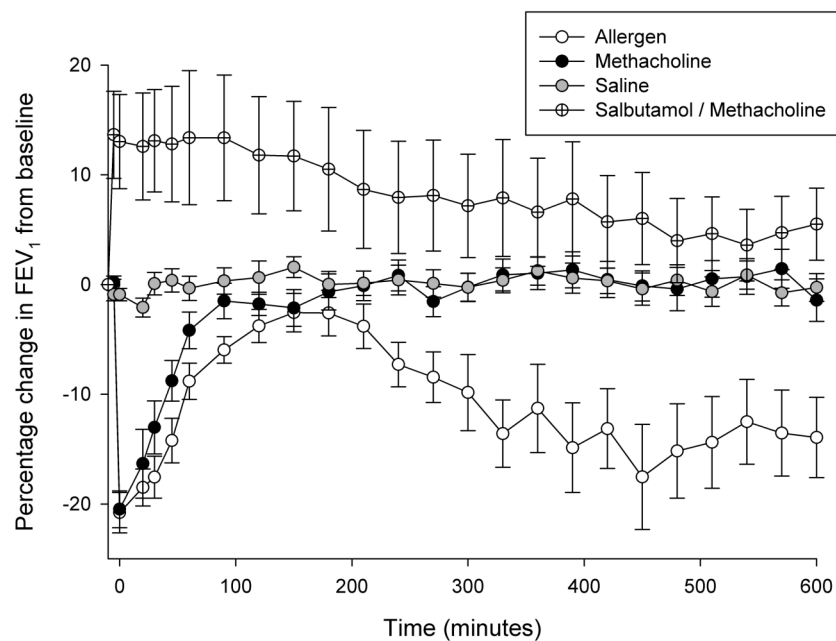


Figure 3-12 Percentage change in FEV₁ over time following third of three allergen, methacholine, saline and salbutamol / methacholine challenges. Mean $\pm$ SEM.

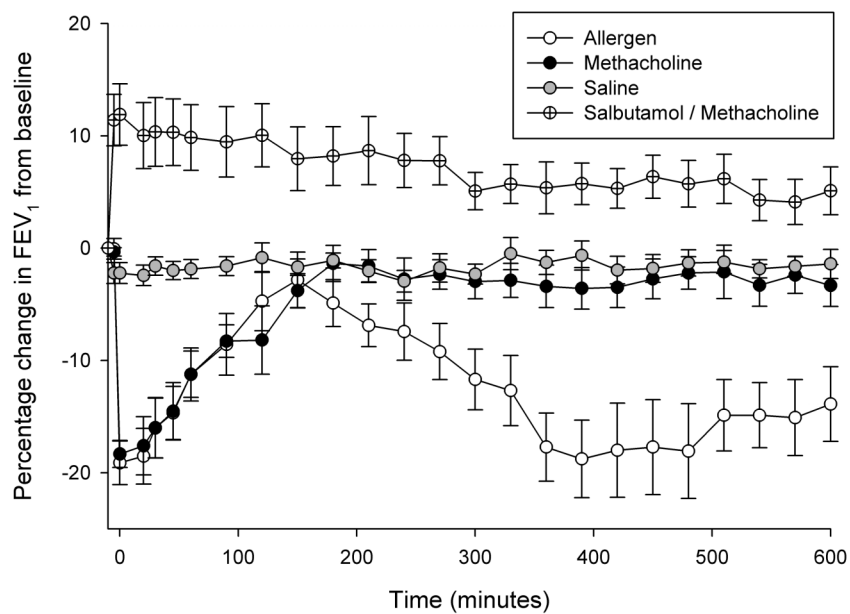


Figure 3-13 Percentage change in FEV₁ following repeated allergen challenge. Mean $\pm$ SEM

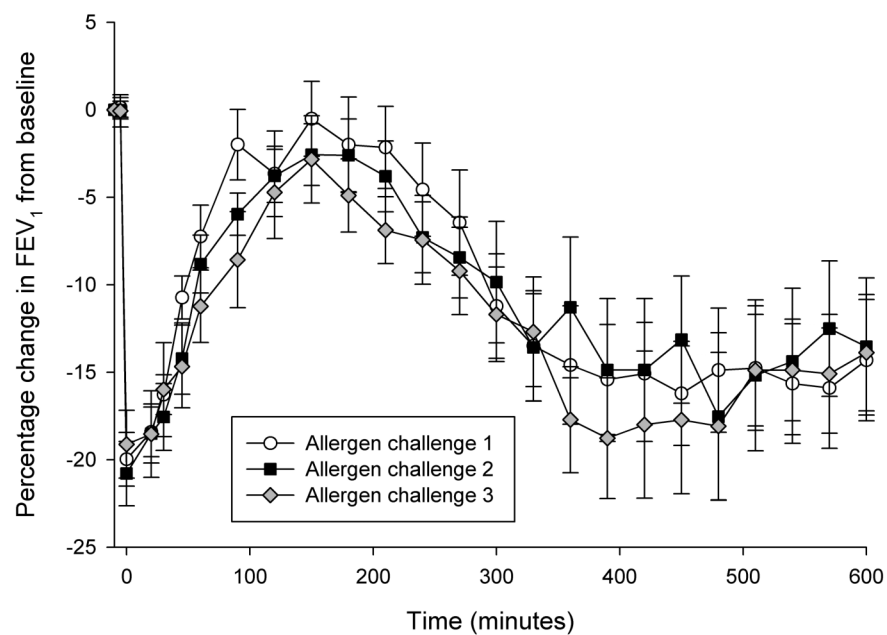


Figure 3-14 Percentage change in FEV₁ following repeated methacholine challenge. Mean $\pm$ SEM

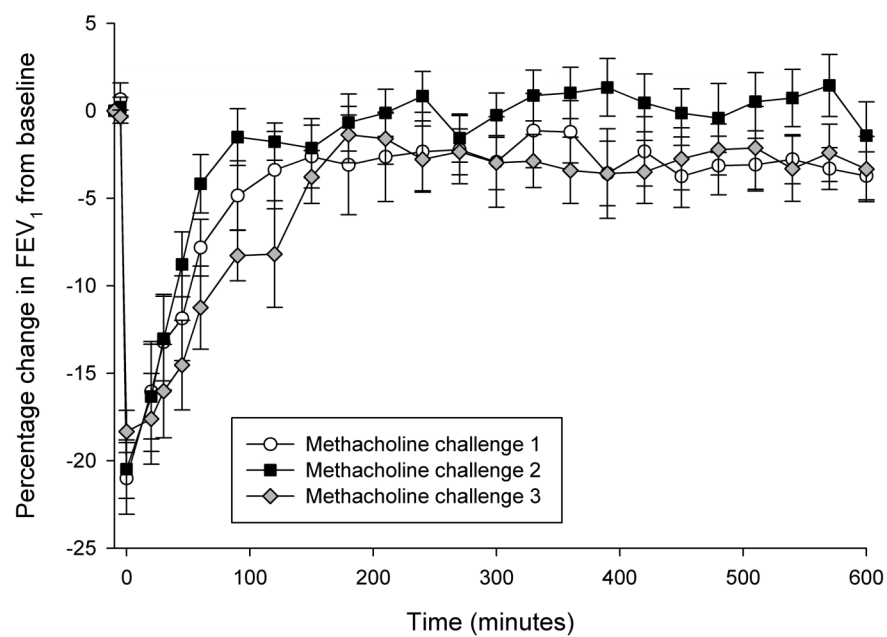


Figure 3-15 Percentage change in FEV₁ following repeated saline challenge. Mean $\pm$ SEM.

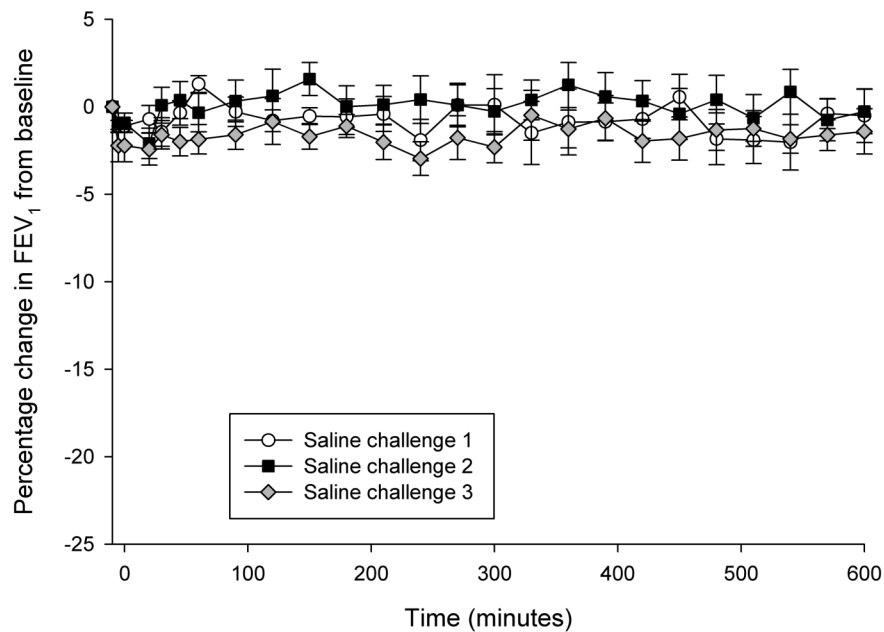


Figure 3-16 Percentage change in FEV₁ following repeated salbutamol and methacholine challenge. Mean $\pm$ SEM.

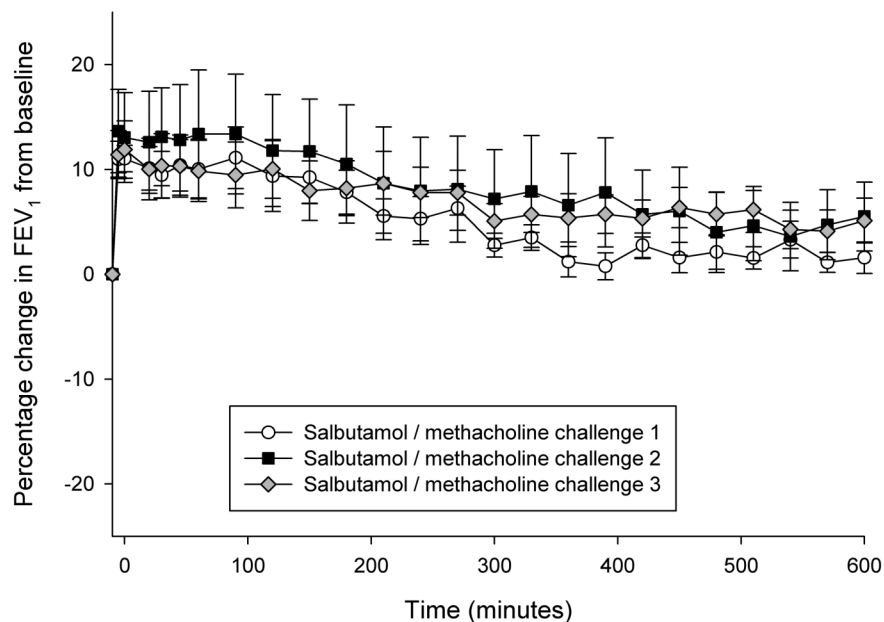
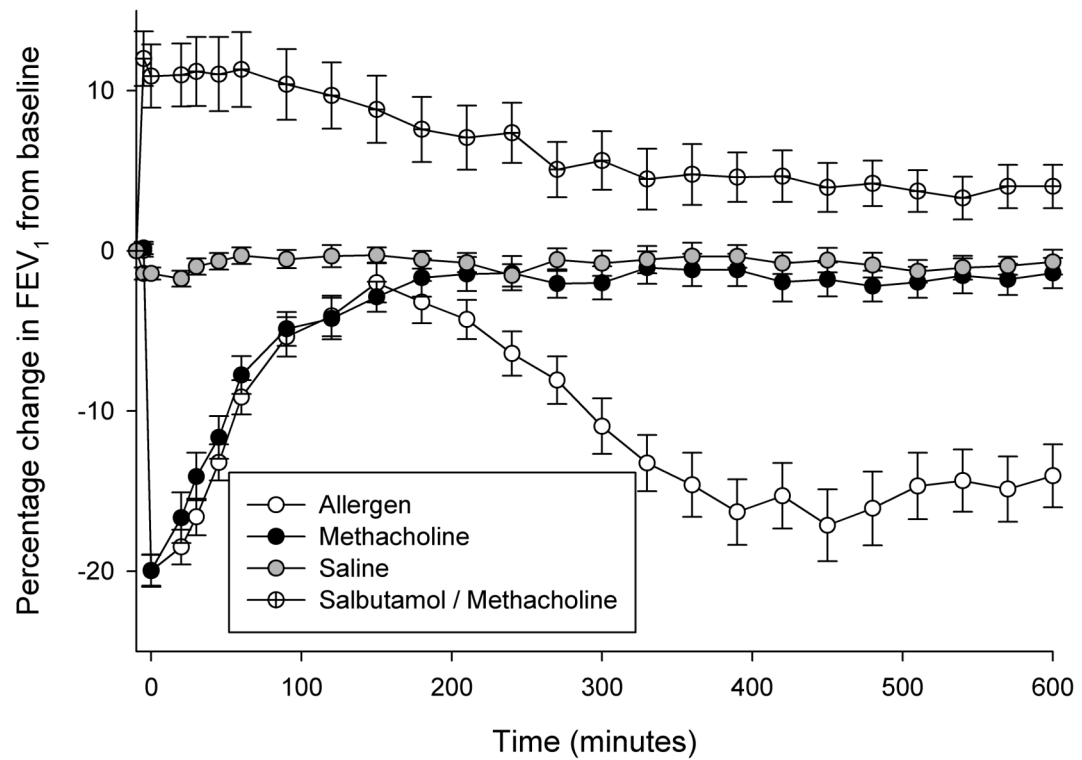


Figure 3-17 Mean change in FEV₁ following three repeated allergen, methacholine, saline and salbutamol / methacholine challenges each 48 hours apart. Mean $\pm$ SEM.



3.3.6 Recovery of FEV₁ prior to repeated challenge

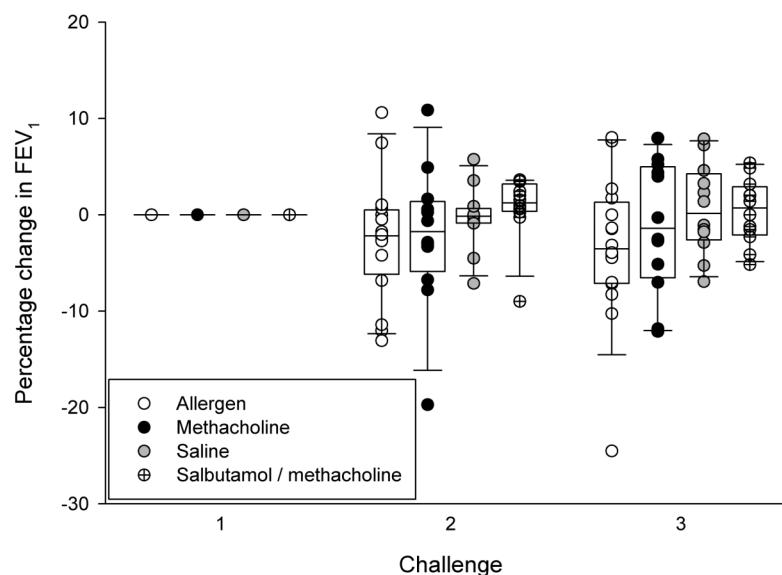
Repeated challenge could result in a gradual reduction in baseline FEV₁ over time. There was a trend towards a decrease in mean pre saline baseline FEV₁ as a percentage of predicted FEV₁ prior to each repeated allergen challenge (91.1%(12.6), 88.7(12.0) and 87.7(12.2) (p=0.052). There was no such trend in any of the other challenges; methacholine 94.0(16.3), 92.2 (13.9) and 93.6(14.3) (p=0.56), saline 93.2(14.4), 92.8(13.6) and 94.6(14.8) (p=0.52) and salbutamol/methacholine 89.2 (13.5), 89.2 (19.1) and 89.8(17.7) (p=0.92). There was no significant difference between the mean pre saline baseline FEV₁ as percentage of predicated between the provocant groups at any of the challenges (p=0.81, 0.84 and 0.61 for the 3 challenges respectively). These results are summarised in Table 3-7 and shown at Figure 3-18.

Table 3-7 Resting mean percentage predicted FEV₁ prior to repeated allergen, methacholine, saline and salbutamol / methacholine challenges.

	Allergen (n=16)	Methacholine (n=12)	Saline (n=12)	Salbutamol (n=12)	p value
Challenge 1	91.1 (12.6)	94.0 (16.3)	93.2 (14.4)	89.2 (13.5)	0.81
Challenge 2	88.7 (12.0)	92.2 (13.9)	92.8 (13.6)	89.2 (19.1)	0.84
Challenge 3	87.7 (12.2)	93.6 (14.3)	94.6 (14.8)	89.8 (17.7)	0.61
p value within group	0.052	0.56	0.52	0.92	

Values are means with SD in parentheses. Values are % predicted FEV₁ before saline challenge on each of the challenge days. p values are calculated by comparison of means using Friedmans test within provocant groups and Kruskal Wallis between groups.

Figure 3-18 Percentage change in FEV₁ prior to repeated allergen, methacholine, saline or salbutamol / methacholine challenges.



3.3.7 Dose of provocant required to cause FEV₁ drop

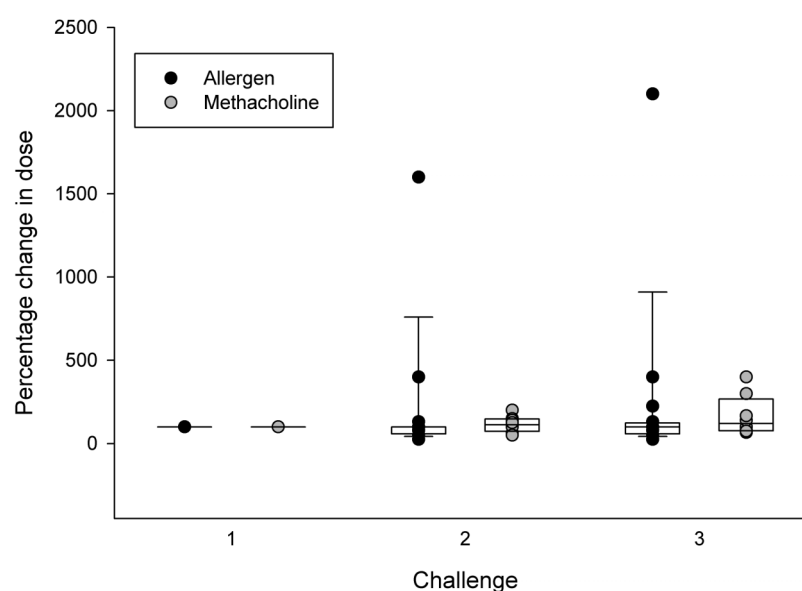
The dose of provocant required to induce the target EAR was recorded and is expressed as a percentage of the dose required to cause the target FEV₁ drop in the initial challenge. In the allergen group the mean (SD) doses administered were 100%(0.0), 199% (382.7) and 238% (504.3) and in the methacholine group the doses administered were 100% (0.0), 144% (84.7) and 234% (291.8). There were no significant within provocant group differences ($p=0.66$ and 0.36 for allergen and methacholine respectively). These results are summarised in Table 3-8. There were some marked individual differences, with one subject in the allergen group showing significant desensitisation; this can be seen at Figure 3-19.

Table 3-8 Change in dose of provocant required to induce 15% drop in FEV₁ on each challenge day.

	Dose of provocant			p
	Challenge 1	Challenge 2	Challenge 3	
Allergen (n=16)	100 (0.0)	199 (382.7)	238 (504.3)	0.66
Methacholine (n=12)	100 (0.0)	144 (84.7)	234 (291.8)	0.36

Values are means with SD in parentheses. Values are percentage of dose required to induce 15% drop in FEV₁ on initial challenge day. p values are calculated by comparison of means using Friedman test.

Figure 3-19 Inhaled doses of allergen or methacholine required to induce a 15% fall in FEV₁ on three consecutive inhalation challenges each separated by 48 hours.



3.3.8 Symptom score

Symptom scores were recorded in the week preceding and in the week of repeated challenges as described in Chapter 2. Mean (SD) daily symptom score for the week preceding repeated challenges was 0.9 (1.6) in the allergen group, 1.4 (1.8) in the methacholine group, 0.4 (0.4) in saline group and 1.2 (1.2) in the salbutamol group ($p=0.22$). Mean (SD) daily scores in the week of the repeated challenges were 3.4 (2.7) in the allergen group, 2.7 (1.6) in the methacholine group, 0.7 (1.1) in the saline and 1.1 (1.3) in the salbutamol group. There was a significant increase in the symptom score only in the allergen group ($p=0.002$) whilst there was a trend towards an increase in the methacholine group ($p=0.054$). In the week of challenge there was a significant difference in the symptom scores when comparing all 4 groups ($p=0.001$). These data are summarised in Table 3-9 and in Figure 3-20. If the change (delta) of the symptom score is considered, then mean (SD) change between the week preceding and the week of challenges was 2.5 (2.3) in the allergen group, 1.3 (2.3) in the methacholine group, 0.4 (0.8) in the saline group and -0.1 (1.4) in the salbutamol group. There was no significant difference between the changes when calculated using Kruskal Wallis test ($p=0.265$) as shown in Table 3-10.

Table 3-9 Mean daily symptom score in week before and week of repeated inhaled challenges.

	Mean symptom scores		p
	Week before repeated challenges	Week of repeated challenges	
Allergen (n=16)	0.9 (1.6)	3.4 (2.7)	0.002
Methacholine (n=12)	1.4 (1.8)	2.7 (1.6)	0.054
Saline (n=12)	0.4 (0.4)	0.7 (1.1)	0.12
Salbutamol (n=12)	1.2 (1.2)	1.1 (1.3)	0.86
p value between groups	0.22	0.001	

Symptom scores in arbitrary units. Values are means with SD in parentheses. p values within provocation groups calculated by Wilcoxon Signed Rank test, between groups differences by Kruskal Wallis Test.

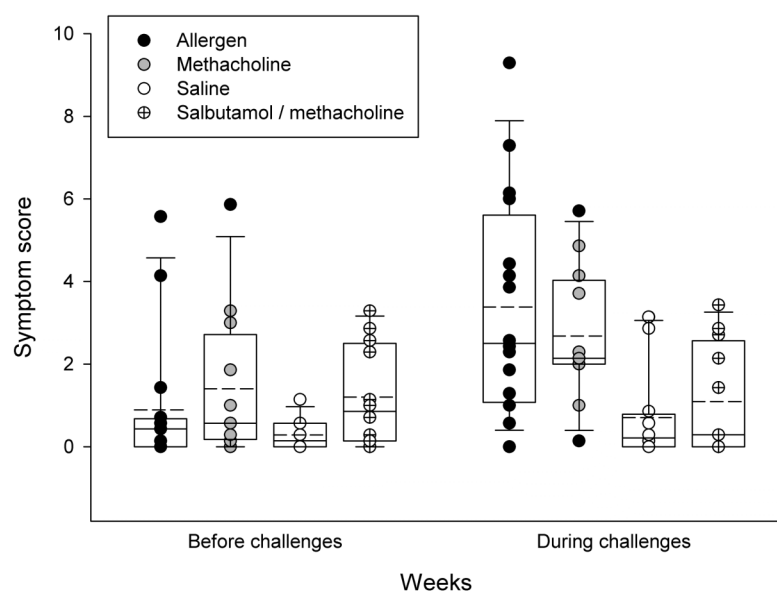
Figure 3-20 Mean daily symptom scores in weeks prior to and during repeated allergen, methacholine, saline or salbutamol / methacholine challenges.

Table 3-10 Mean change in symptom score and daily reliever use between week prior to and week of repeated allergen, methacholine, saline, and salbutamol / methacholine challenges.

	Δ Symptom score	Δ reliever use score
Allergen (n=16)	2.5 (2.3)	1.4 (1.9)
Methacholine (n=12)	1.3 (2.3)	0.01 (0.8)
Saline (n=12)	0.4 (0.8)	-0.04 (0.3)
Salbutamol (n=12)	-0.1 (1.4)	0.04 (0.8)
p value between groups	0.27	0.007

Change in symptom score and daily reliever between week prior to and week of repeated inhaled challenges. Values are mean with SD in parentheses. p values calculated using Kruskal Wallis test.

3.3.9 Reliever use

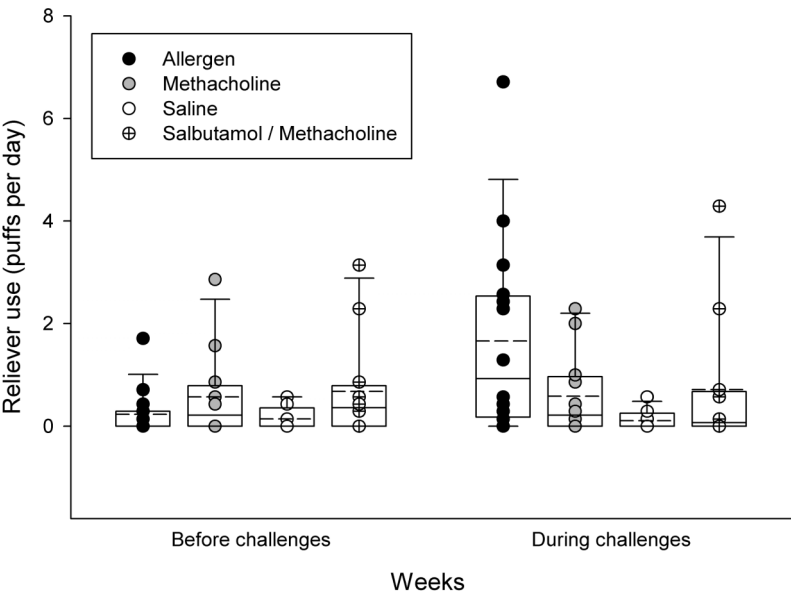
Daily reliever use in terms of puffs per day was recorded in the week preceding and in the week of repeated challenges as described in Chapter 2. Mean daily reliever use for the week preceding repeated challenges was 0.2 (0.4), 0.6(0.9), 0.2(0.3) and 0.7 (1.0) ($p=0.42$ for difference) in the allergen, methacholine, saline and salbutamol / methacholine groups respectively. There was a significant increase in the amount of reliever medication used in the allergen group, increasing to 1.7 (1.9) puffs per day ($p=0.003$). In the other three groups there was no increase, the mean (SD) being 0.6 (0.8), 0.1(0.2) and 0.7 (1.3) in the three remaining groups. There was a significant between group difference for the week of challenges ($p=0.009$). These data are summarised in Table 3-11 and graphically in Figure 3-21. If the change (delta) of the daily reliever use is considered, then mean (SD) change between the week preceding and the week of challenges was 1.4 (1.9) in the allergen group, 0.01 (0.8) in the methacholine group, -0.04 (0.3) in the saline group and 0.04 (0.8) in the salbutamol group. There was a significant between group difference ($p=0.007$). These data are shown in Table 3-10.

Table 3-11 Mean daily reliever use in week before and week of repeated inhaled challenges.

	Mean daily reliever use		p
	Week before repeated challenges	Week of repeated challenges	
Allergen (n=16)	0.2 (0.4)	1.7 (1.9)	0.003
Methacholine (n=12)	0.6 (0.9)	0.6 (0.8)	0.41
Saline (n=12)	0.2 (0.3)	0.1 (0.2)	0.67
Salbutamol (n=12)	0.7 (1.0)	0.7 (1.3)	0.92
p value between groups	0.42	0.009	

Mean Reliever use in units of puffs per day. Values are means with SD in parentheses. p values within provocation groups calculated by Wilcoxon Signed Rank test, between groups differences by Kruskal Wallis Test.

Figure 3-21 Mean daily reliever use in weeks prior to and during repeated allergen, methacholine, saline or salbutamol / methacholine challenges.



3.4 Discussion

These series of experiments have demonstrated that repeated high dose allergen challenge in human volunteers with mild asthma is safe and acceptable. There were no significant adverse events requiring hospital admission or rescue anti-inflammatory medication.

High dose repeated allergen challenge has been performed in human volunteers previously (de Bruin-Weller 1996). In this small study of eight volunteers the authors administered allergen by inhalation, targeting a fall in FEV₁ of 15% from baseline with a single repeat challenge at an interval of 48 hours. In this group they noted an EAR in all patients, and a LAR in six following the first allergen challenge, and in seven on the second occasion. The authors also noted that repeated challenge did not result in significant changes in the severity of the EAR or LAR; they did however note a decrease in the PC₂₀ to histamine following the first challenge, but no additional drop following the second challenge.

Daily high dose allergen challenge has also been performed for four successive days, though in 1975, with the aim of examining the effects of repeated allergen exposure on priming or desensitisation (Rosenthal 1975). Using ragweed extract, no regular trend towards either effect was found, though some individuals showed such changes (3 priming, 3 desensitisation from total study group of 13). In this study a 35% drop in airways conductance was the target, with the dose required to achieve this being recorded. Only 2 late reactions were noted, but these were recorded on the basis of clinical symptoms only.

Recently it has been demonstrated that HDM and grass pollen have different effects on the LAR when matched for a similar EAR, and hence the findings of Rosenthal cannot be extrapolated to repeated allergen challenge with HDM

extract (Hatzivlassiou 2010). In the study above (de Brui-Weller 1996) of the 8 subjects, 2 were challenged with grass pollen and 6 with house dust mite (de Bruin-Weller 1996).

When high dose inhaled allergen challenge has been performed at a longer interval, such as two weeks, Rasmussen *et al* demonstrated no change in the EAR, but a significantly greater fall in FEV₁ during the LAR. In this study the target EAR FEV₁ drop was 20%, however the mean fall generated was 35%. The allergens used in this study were primarily grass or birch pollen, with a few subjects exposed to cat or dog dander, again making the extrapolation of results to HDM difficult.

Though repeated allergen challenge is uncommon, and the specific protocol employed in this study has not been examined previously, there is a large body of experience of single high dose allergen challenges. Following these challenges there is an increase in non specific airway hyperreactivity as measured by methacholine or histamine challenges (Kariyawasam 2007, Cockroft 1977). This is generally thought to be correlated with the presence of a LAR (rather than an isolated EAR) inducing this increase in reactivity. The original work from Cockroft *et al* in 1977 which is often quoted only reported 7 patients with an increase in non specific reactivity, of which only 3 had late falls in FEV₁ of between 5 and 11% (Cockroft 1977). More recent work has shown that single allergen challenge with a LAR induces an increase in AHR to methacholine at 24 hours, which is still detectable at 7 days, however AHR following allergen challenge with only an EAR has not been recently investigated (Kariyawasam 2007).

On the basis of this previous work, it might have been expected that with an increase in non-specific AHR likely to be present following allergen challenge with induction of a LAR, the dose of allergen required to induce a 15% fall in FEV₁ might fall over the three challenge days in our model. This was not the

case, with almost all subjects requiring the same dose of allergen, and no significant change in dose required. On the contrary, 2 subjects showed marked desensitisation, and required increased doses. These findings imply that although allergen challenge with induction of an LAR is likely to induce non-specific AHR, this non-specific response is not triggered by the repeated inhalation of allergen at a 48 hour interval.

Though there was no difference in the magnitude of the EAR with repeated allergen challenge when measured by maximal FEV₁ fall, or area under the curve calculations for up to 120 minutes, there was a trend towards increased duration of the EAR as the AUC increased from 1161 through 1302 to 1510 in the three allergen challenges ($p=0.07$). This may be an indication of the presence of increased airway hyperreactivity, manifesting in terms of increased duration of EAR, rather than its absolute magnitude. There was no difference in the magnitude of the LAR by either measurement over the course of the three challenges.

LAR can be increased by increasing dose of allergen (Lai 1989) and in the patient who had the large increase in the allergen dose, this was indeed the case, with maximal LAR going from 11.3% at the first challenge to 28.7% at the second and 23.1% in the final challenge. The AUC similarly increased from 2523 to 8744 and 7592.

It is possible that our initial allergen challenge did not induce non-specific AHR, and the (not statistically significant) trend increase in EAR AUC occurred by chance, rather than due to increased AHR. Previous studies which have noted increased AHR have often induced a greater EAR FEV₁ fall than achieved here. Our mean (SD) FEV₁ fall was 20.7 % (6.5) in the first allergen challenges, whilst another study which examined AHR at 24 hours and 7 days induced a fall of 35%(11.7) (Kariwayasam, 2007). The magnitude of the EAR may have an impact on the degree of non-specific AHR. In order to investigate

this further it would be necessary to repeat the study with methacholine or histamine challenges on the days after the allergen challenges. This was not carried out in this study, to prevent possible confounding from the presence of repeated bronchoconstriction.

3.4.1 Repeated methacholine challenges

Repeated methacholine challenge in normal individuals, but not in asthmatics, has been shown to cause an attenuated airway response to the same dose of methacholine administered (Stevens 1990, Beckett 1992). In keeping with these findings there was no change in the dose of methacholine required to induce a 15% drop in FEV₁ between the three methacholine challenges, though there was a non significant trend towards an increased dose being required. The repeated methacholine challenges were matched to a target fall in FEV₁, and this was achieved with no significant differences between the FEV₁ fall obtained over the 3 challenges; there was also no change in the AUC following the three challenges up to 120 mins (termed EAR for ease of explanation, though no allergic response was induced).

3.4.2 Repeated saline challenges

As expected, the saline challenges resulted in no changes in FEV₁ measured either by maximal FEV₁ change or area under the curve for either of the EAR or LAR time periods.

3.4.3 Repeated salbutamol / methacholine challenges

Administration of salbutamol, as expected, lead to a significant increase in FEV₁ from baseline; subsequent administration of twice the concentration of methacholine required to induce a 20% fall in FEV₁ at screening did not change the FEV₁ significantly. The aim of completely blocking the bronchconstrictive response from the methacholine was therefore achieved, and this group can be

used to assess whether any airway changes occurred in the methacholine group as a response to bronchoconstriction alone, rather than as a response to direct stimulation of M2 or M3 receptors.

3.4.4 FEV₁ prior to repeated challenge

With repeated allergen challenge there is a concern that over the course of the challenges, subjects respiratory function may gradually worsen, so making the repeated challenges unsafe. In order to proceed onto an allergen challenge, each subject required an FEV₁ >70% of predicted on the morning of the challenge test, this was achieved in each case. Prior to each repeated challenge there was no statistically significant difference in FEV₁ recorded in any group, though in the allergen challenge group there was a trend towards significance with the pretest FEV₁ decreasing from mean 91.1% to mean of 87.7% by the last challenge ($p=0.052$). Were further allergen challenges planned, this trend towards a reduction in FEV₁ could become both statistically and clinically significant, probably resulting in a limit in the number of allergen challenges that could safely be performed.

3.4.5 Symptom scores and Reliever use

Symptom scores from the subjects were significantly increased only in the allergen group, although there was a trend ($p=0.054$) towards an increase in the methacholine group. The similarity of the symptom scores might suggest that the symptoms are due primarily to the early fall in FEV₁, rather than the presence of any LAR – ie the LAR might be relatively symptom free. Early work based purely on symptoms certainly underestimated the frequency of the LAR due to its relative lack of symptoms (Herxheimer 1952). An alternative explanation is that symptoms and the degree of airway obstruction are not well correlated; this has been shown to be the case following methacholine challenge (Mansur 2008, Tetzlaff 1999, Weiner 2003).

Reliever medication use was significantly higher in the allergen group, this may be a reflection of the open label nature of the study; subjects were expecting to become more symptomatic from the allergen exposure, and therefore self administered more medication. There was an increase in both symptoms and reliever use, which are in keeping with the falls in FEV₁ experienced.

3.4.6 Further work

As a result of the above work, it is now possible to make an assessment of the airway responses to challenges which induce similar early bronchoconstrictor responses (allergen and methacholine) and those which do not induce early bronchoconstriction (saline and salbutamol followed by methacholine). In the next chapter, the inflammatory responses to these four airway challenges will be investigated.

Chapter 4 Inflammation as a result of repeated inhalation challenges

In the previous chapter we demonstrated a safe, repeatable and acceptable method for exposing an individual to inhalation challenge with either methacholine or allergen, to induce a matched early bronchoconstriction in both groups. In addition, control groups were included which demonstrated that there was no fall in FEV₁ or change in symptoms or reliever medication use from either repeated saline or salbutamol followed by methacholine inhalation.

In order to investigate further whether the remodelling response in asthma is due to an eosinophilic inflammatory response or due to bronchoconstriction in the absence of eosinophilic infiltration of the airway, the next step was to assess the subjects who underwent repeated bronchoscopy as to the degree of inflammation induced by the repeated inhalation challenges.

4.1 Background

Previous published work has demonstrated that single inhaled allergen challenge in human volunteers results in both cellular inflammation and remodelling changes when examined by repeated bronchoscopy at 24 hours following allergen inhalation (Kariyawasam 2007). By seven days following allergen challenge however, the inflammatory response has resolved, though some remodelling changes and airway hyperreactivity persist (Kariyawasam 2007). This study did not examine the possibility that repeated bronchoscopy by and of itself could induce either inflammatory responses or remodelling. Also, the authors incorporated into the study design repeated methacholine challenges to assess bronchial hyperreactivity, so inducing repeated bronchoconstriction. This repeated bronchoconstriction could itself induce airway inflammation by several mechanisms, including a reduction of

intercellular volume in the constricted epithelium (Halldorsdottir 1997, Tschumperlin 2004).

Although it is well established that cellular inflammation following an inhaled allergen challenge is present 24 hours after challenge, in both experimental and environmental allergen exposure (Kämpe 2010, van Rensen 2009) it has not been established how long this inflammation persists for, excepting the study above which studied inflammation seven days after a single challenge.

Although the effects of allergen challenge on inflammation within the airway have been examined extensively, there has been little work examining the potential inflammatory effects of methacholine challenge on airway inflammation. Methacholine certainly affects the responses of the airway; methacholine challenge prior to mannitol challenge blunts the subsequent response to mannitol, though in a manner that is not currently understood (Gade 2009). It is also known that repeated methacholine testing in asthmatic individuals does not induce tolerance to the methacholine, and this lack of tolerance (which is seen in non asthmatics) has been attributed to underlying (rather than methacholine induced) inflammation, with perhaps an increased clearance of the methacholine in the inflamed lung (Beckett 1992, Stevens 1990). This may not be the case, as in animal models the degree of attenuation of response to methacholine is not associated with the level of inflammation (McKenzie 2008). The mechanisms by which methacholine administration affects airway hyperreactivity are not known, but from these studies it is apparent that methacholine induces changes to AHR, and others have shown that it affects the airway directly. Methacholine administered in the nose has been shown to induce secretion of fucose (used as a marker of mucin secretion) (Grieff 2005) and also a small increase in sputum α 2-macroglobulin when administered by inhalation (Halldorsdottir 1997), although a single methacholine challenge in normal people showed no change in cellular composition of the BAL at 24 hours (Söderberg 1993). The effects of

methacholine on the airway of normal individuals may be significantly different to those in asthmatics. There are no studies which examine potential inflammatory changes following methacholine inhalation in asthmatics either with respect to BAL cellular constituents, or cellular inflammation within the tissue at bronchial biopsy, however induced sputum from a small number of asthmatics exposed to methacholine has shown no eosinophilic inflammatory response (Gauvreau 2000). Our repeated challenge protocol offers an opportunity to assess any possible changes in cellular composition of the airway following repeated methacholine challenge, as the repeated nature of the challenge may provide increased stimulus for any changes and hence increased sensitivity to any changes that result. The protocol also allows any changes that may occur in the methacholine group to be compared to any changes in the salbutamol / methacholine group to establish if such changes are a result of repeated bronchoconstriction or direct chemical effects of methacholine administration.

The degree of cellular inflammation that is present 4 days after multiple allergen and other inhalation challenges is currently unknown, and may be examined in various ways; by the assessment of the degree of cellular inflammation in bronchial biopsies, the cellular composition of the BAL fluid, and the presence and concentrations of inflammatory mediators in the BAL fluid such as interleukin 8 and eosinophil cationic protein.

4.1.1 Interleukin 8

Interleukin 8 (IL8) is a major mediator of the cellular inflammatory response and has been implicated in the neutrophilic inflammation that is present in some severe asthmatics (Simpson 2007). Although IL8 is produced in small quantities by most nucleated cells in the body, the majority of its secretion is from monocytes and macrophages, though in the airway the respiratory epithelial cell may play an important secretory role (Remick 2005). IL8 is a small protein of

only 72 amino acids, and is a member of the CXC group of cytokines, where C represents a cysteine amino acid, X another amino acid moiety, and the final C another cysteine residue; as IL8 is a ligand, under the CXC nomenclature it is referred to as CXCL8. IL8 is produced in response to multiple stimuli within the respiratory tract, including viral infection with rhinovirus, and provocation with house dust mite antigen, as well as exposure to bacteria associated molecular patterns (Bossios 2008). Following the initial release of IL8, unlike many other cytokines, its secretion continues for many hours, days or even weeks following the inflammatory stimulus, making it useful in the delayed measurement in this study. Its release, acting via specific G protein coupled receptors (CXCR1 and CXCR2) results in the chemotaxis of neutrophils along a chemotactic gradient, but also induces other effects associated with inflammation, such as angiogenesis and, in animals at least, increased bronchial hyperresponsiveness (Fujimura 1996). Interestingly IL8 levels in BAL fluid from patients with severe asthma are similar to those with less severe disease (Macedo 2009). It is expected that IL8 levels in BAL would increase following allergen challenge, but it has not been investigated with the specific allergen challenge protocol used here, nor have IL8 levels been examined following repeated bronchoconstriction which could of itself induce an inflammatory response.

4.1.2 Eosinophil cationic protein

Eosinophil cationic protein (ECP) was originally purified from cells obtained from patients with chronic myeloid leukaemia; only later was it identified as eosinophilic in origin. The protein is found in specific granules within eosinophils along with three other proteins; eosinophil protein X, eosinophil peroxidase and major basic protein. Normal cells contain approximately 10 μ g of ECP per 10⁶ cells, though cells from atopic individuals may both contain and release more (Venge 1999). ECP release from eosinophils is triggered by various stimuli including IgG complexes, interleukin 5 and platelet activating factor (Koh 2007). The protein, as its name suggests, is highly charged and varies in molecular weight due to variable glycosylation which can occur at three different points on

the amino acid backbone. The significant charge results in the protein being highly bound, especially to negatively charged molecules such as those found in the cell membrane; this binding may provide a mechanism by which its actions are regulated by basic proteins such as heparin and α 2-macroglobulin (Venge 1999). ECP has various effects when released during eosinophilic degranulation; these effects may be considered as cytotoxic and non cytotoxic effects. ECP exerts its cytotoxic effects against bacteria, parasites and also respiratory epithelial cells by forming pores within the cell membrane that result in osmotic mediated cell lysis. Non cytotoxic effects include an increase in mucus secretion within the airway, as well as upregulating insulin like growth factor 1 (IGF1) receptor and intercellular adhesion molecule (ICAM) expression in epithelial cells, in addition to activating an inflammatory cascade resulting in mast cell activation and histamine release (Koh 2007).

ECP is the most commonly used marker for eosinophilic inflammation in asthma, and is often identified using the monoclonal antibody to ECP, EG2. This antibody was originally thought to distinguish between the active and inactive eosinophil (Tai 1984) but now this appears to be more dependent on cell fixation methods than cellular activation (Jahnsen 1994).

In asthma, BAL ECP is found to be associated with the presence of the late allergic reaction following allergen challenge both in the laboratory and with allergen exposure in the environment (De Monch 1985). There is an association of serum and BAL ECP levels, and also a correlation between ECP levels, numbers of eosinophils in BAL and the severity of asthma (Crimi 1998, Bousquet 1990). ECP has been recently shown to be chemotactic to human lung fibroblasts *in vitro*, establishing another possible mechanisms for its role in airway remodelling (Zagai 2009), and a faster decline in lung function in asthma is associated with increased eosinophil numbers and ECP in the sputum (Broekema 2010).

With the repeated inhalation challenge model presented here, an increase in both BAL ECP levels and the number of EG2 staining eosinophils in the tissue would be expected in the allergen challenge group.

4.2 Aims

To establish the effect of repeated inhalation challenges performed as previously described on luminal and tissue inflammatory responses four days after the final inhalation challenge. To test the hypothesis that repeated allergen, methacholine, saline and salbutamol followed by methacholine challenges would induce similar inflammatory responses within the airway lumen and tissue.

4.3 Methods and results

4.3.1 Volunteers

The same volunteers which were recruited, characterised and challenged as described in Chapter 2 were examined in this part of the study except where explicitly stated.

4.3.2 Repeated bronchoscopy

All volunteers who undertook the first of the two bronchoscopies completed their inhalation challenges, however it was not possible to complete the repeat bronchoscopy in all individuals. 16 subjects underwent repeated inhaled allergen challenge, whilst 12 subjects underwent repeated challenge with either methacholine, saline or salbutamol followed by methacholine as previously described. Of the 16 volunteers undergoing repeated allergen challenge, it was possible to perform repeated bronchoscopy on only 12. All volunteers in the other challenge groups completed both bronchoscopies. This difference was significant, $p=0.021$ by Fishers exact test.

Comparing the allergen challenged individuals who were able to complete the paired bronchoscopies, with those who were unable to complete them, there were no significant differences in any baseline characteristic except age (both bronchoscopy group mean (SD) age in years 23.4 (2.5) and single bronchoscopy group 26.0 (9.3) $p=0.04$) as shown at Table 4-1. It might be postulated that the group unable to tolerate repeated bronchoscopy reacted more strongly in some manner to the inhaled allergen than the bronchoscopy tolerating other group, however there was no difference in the mean maximal fall in FEV₁ or area under the curve for spirometry during the early and late asthmatic reactions between the two groups (Table 4-2 and Table 4-3). The pre challenge FEV₁ in the allergen challenge group trended towards a fall in FEV₁ when considered as a whole as described above, and it could have been that the failed bronchoscopy subgroup had a greater fall in FEV₁ than the repeated bronchoscopy subgroup, however the opposite was the case. As shown in Table 4-4, the repeated bronchoscopy group showed a significant reduction in percentage predicted FEV₁ prior to challenge over the course of the three challenges falling from a mean (SD) of 89.1 (13.0)% prior to the first challenge to 85.4 (11.4) prior to the second challenge and 84.5 (12.2) prior to the third challenge ($p=0.03$). The failed bronchoscopy subgroup did not have such a fall, with mean (SD) pre challenge percentage predicted FEV₁ of 97.1 (10.5), 98.4 (8.6) and 97.1 (6.4) respectively. There were also no significant differences between the subgroups in terms of symptom score or reliever use (Table 4-5 and Table 4-6).

Table 4-1 Subject characteristics of volunteers undergoing repeated inhaled allergen challenges who were able and unable to tolerate repeated bronchoscopy.

	Completed repeated bronchoscopy (n=12)	Failed repeat bronchoscopy (n=4)	p value
Sex (M/F)	3/8	1/3	0.83
Age (years)	23.4 (2.5)	26.0 (9.3)	0.04
% Predicted FEV ₁	94.5 (9.7)	108.7 (7.3)	0.68
% Predicted FVC	100.6 (9.9)	112.4 (6.0)	0.37
PC ₂₀ methacholine	1.1 (0.5 - 2.2)	1.2 (0.4 - 4.6)	0.95
BMI	23.7 (1.5)	25.0 (3.7)	0.07
Skin prick test wheal diameter (Histamine)	4.3 (1.5)	3.6 (1.4)	0.77
Skin prick test wheal diameter (HDM)	7.3 (1.5)	5.3 (1.8)	0.48

Values are means with SD in parentheses, except for PC₂₀ methacholine which is median (IQR). p values are calculated by comparison of means using independent samples T test except for PC₂₀ which uses Mann-Whitney Test, and sex, which is calculated by Fishers exact test.

Table 4-2 Mean maximal falls in FEV₁ during early and late asthmatic responses in volunteers undergoing repeated inhaled allergen challenge who were able and unable to tolerate repeated bronchoscopy.

	Completed repeated bronchoscopy (n=12)	Failed repeated bronchoscopy (n=4)	p value
FEV ₁ EAR challenge 1	-20.8 (6.3)	-21.2 (5.0)	1.00
FEV ₁ EAR challenge 2	-22.3 (7.6)	-23.2 (5.7)	0.60
FEV ₁ EAR challenge 3	-20.5 (9.1)	-25.3 (4.0)	0.38
p value within group	0.92	0.37	
FEV ₁ LAR challenge 1	-23.1 (12.4)	-21.8 (18.5)	0.95
FEV ₁ LAR challenge 2	-25.4 (15.2)	-20.4 (12.6)	0.68
FEV ₁ LAR challenge 3	-27.5 (14.4)	-21.8 (18.5)	0.68
p value within group	0.17	0.36	

Values are means with SD in parentheses. EAR = lowest FEV₁ measured during Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). p values are calculated using Mann-Whitney Test between groups and Friedman's Test within groups.

Table 4-3 Mean area under the curve for changes in FEV₁ during early and late asthmatic responses in volunteers undergoing repeated inhaled allergen challenge who were able and unable to tolerate repeated bronchoscopy.

	Completed repeated bronchoscopy (n=12)	Failed repeated bronchoscopy (n=4)	p value
AUC EAR challenge 1	1150(437)	1194 (129)	0.68
AUC EAR challenge 2	1370 (533)	1101 (414)	0.26
AUC EAR challenge 3	1530 (792)	1448 (196)	0.60
p value within group	0.05	0.37	
AUC LAR challenge 1	5799 (4044)	4425 (4165)	0.45
AUC LAR challenge 2	6241 (3656)	4800 (4218)	0.52
AUC LAR challenge 3	6601 (3563)	6779 (6466)	1.00
p value within group	0.72	0.37	

Values are means with SD in parentheses. EAR = Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). AUC = area under the curve. p values are calculated using Mann-Whitney Test (between groups) or Friedmans' test (within groups).

Table 4-4 Percentage predicted FEV₁ prior to repeated inhaled allergen challenge in volunteers able and unable to tolerate repeated bronchoscopy.

	Completed repeated bronchoscopy (n=12)	Failed repeated bronchoscopy (n=4)	p value
Challenge 1	89.1 (13.0)	97.1 (10.5)	0.26
Challenge 2	85.4 (11.4)	98.4 (8.6)	0.08
Challenge 3	84.5 (12.2)	97.1 (6.4)	0.10
p value within group	0.03	0.78	

Values are means with SD in parentheses. Values are % predicted FEV₁ before saline challenge on each of the challenge days. p values are calculated by comparison of means using Friedmans test within provocation groups and Mann-Whitney Test between groups.

Table 4-5 Mean daily symptom scores in volunteers undergoing repeated allergen challenge who were able and unable to tolerate repeated bronchoscopy.

	Mean daily symptom score		p within group
	Week before repeated challenges	Week of repeated challenges	
Completed repeated bronchoscopy (n=12)	1.2 (1.8)	3.6 (2.6)	0.008
Failed second bronchoscopy (n=4)	0.1 (0.2)	2.6 (3.1)	0.07
p value between groups	0.10	0.45	

Symptom scores in arbitrary units. Values are means with SD in parentheses. p values within provocation groups calculated by Wilcoxon Signed Rank test, between groups differences by Mann-Whitney Test.

Table 4-6 Mean daily reliever use in volunteers undergoing repeated allergen challenge who were able and unable to tolerate repeated bronchoscopy.

	Mean daily reliever use		p within group
	Week before repeated challenges	Week of repeated challenges	
Completed repeated bronchoscopy (n=12)	0.2 (0.5)	1.4 (1.4)	0.01
Failed second bronchoscopy (n=4)	0.3 (0.3)	2.4 (3.0)	0.11
p value between groups	0.68	0.68	

Mean Reliever use in units of puffs per day. Values are means with SD in parentheses. p values within provocation groups calculated by Wilcoxon Signed Rank test, between groups differences by Mann-Whitney Test.

4.3.3 Challenge spirometry

As not all subjects exposed to repeated inhaled allergen challenge underwent repeated bronchoscopy it was important to establish whether the exclusion of four of the sixteen exposed subjects would have an effect on the overall responses described in the previous chapter. Comparing only volunteers who underwent both of the bronchoscopies in the four inhalation challenge groups, there was no difference in the baseline characteristics as shown in Table 4-7. In addition the spirometry changes following the inhalation challenges were similar to those seen with the larger groups. There was a mean maximal (SD) fall in FEV₁ during the first allergen challenge in the group which had both bronchoscopies of 20.8 (6.3)% in the second challenge of 22.3 (7.6) and of 20.5 (9.1) in the third challenge ($p=0.92$ for within group difference) (Table 4-8). Similar results to those obtained with the group of sixteen were seen for area under the curve (Table 4-9). There was no significant difference between the FEV₁ falls and area under the curve for the spirometry during the EAR between allergen and the methacholine challenge groups, whilst there remained a significant difference between the allergen and methacholine groups and the two control groups. There was a significant difference between the mean maximal drop in FEV₁ / area under the curve between allergen and all other groups including methacholine during the time of the late allergic reaction (Table 4-8, Table 4-9, Table 4-10 and Table 4-11).

Table 4-7 Baseline volunteer characteristics of all subjects tolerating repeated bronchoscopy.

	Allergen (n = 12)	Methacholine (n=12)	Saline (n=12)	Salbutamol (n=12)	p value
Sex (M/F)	3/9	4 / 8	4 / 8	2 / 10	0.73
Age (years)	23.4 (2.5)	25.4 (10.4)	21.0 (3.5)	21.0 (3.5)	0.26
% Predicted FEV ₁	89.1 (13.0)	93.9 (16.3)	93.2 (14.4)	89.2 (13.5)	0.77
% Predicted FVC	100.6 (9.9)	105.5 (16.5)	105.4 (12.4)	108.3 (11.0)	0.52
PC ₂₀ methacholine	1.1 (0.47 - 2.23)	1.3 (0.18 - 3.69)	1.7 (1.1 - 2.7)	2.0 (0.85 - 2.73)	0.64
BMI	23.7 (1.5)	23.6 (3.6)	22.8 (2.6)	22.7 (2.2)	0.71
Skin prick test wheal diameter (Histamine)	4.3 (1.5)	4.2 (1.3)	4.3 (1.3)	4.7 (1.4)	0.84
Skin prick test wheal diameter (HDM)	7.3 (1.5)	6.5 (3.3)	7.4 (3.6)	7.4 (2.4)	0.85

Values are means with SD in parentheses, except for PC₂₀ methacholine which is median (IQR). p values are calculated by comparison of means using ANOVA except for PC₂₀ which uses Kruskal Wallis, and sex, which is calculated by Fishers exact test.

Table 4-8 Mean maximal changes in FEV₁ following repeated inhalation challenges in subjects who completed repeated bronchoscopy.

	Allergen (n=12)	Methacholine (n=12)	Saline (n=12)	Salbutamol (n=12)	p value
FEV ₁ EAR challenge 1	-20.8 (6.3)	-22.1 (7.5)	-5.1 (4.7)	6.9 (7.7)	<0.001
FEV ₁ EAR challenge 2	-22.3 (7.6)	-23.1 (8.3)	-3.9 (3.0)	10.5 (16.1)	<0.001
FEV ₁ EAR challenge 3	-20.5 (9.1)	-22.1 (6.9)	-4.9 (2.9)	7.5 (8.9)	<0.001
p value within group	0.92	0.89	0.37	0.99	
FEV ₁ LAR challenge 1	-23.1 (12.4)	-8.5 (7.0)	-5.1 (4.7)	-1.4 (5.2)	<0.001
FEV ₁ LAR challenge 2	-25.4 (15.2)	-5.7 (3.8)	-4.5 (4.7)	-0.1 (10.0)	<0.001
FEV ₁ LAR challenge 3	-27.5 (14.4)	-9.7 (8.8)	-5.2 (3.1)	-0.6 (3.6)	<0.001
p value within group	0.17	0.54	0.85	0.77	

Values are means with SD in parentheses. EAR = lowest FEV₁ measured during Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). p values are calculated using Kruskal-Wallis test both between groups and Friedman's Test within groups.

Table 4-9 Area under the curve for changes in FEV₁ following repeated inhalation challenges in subjects who completed repeated bronchoscopy.

	Allergen (n=12)	Methacholine (n=12)	Saline (n=12)	p Value
AUC EAR challenge 1	1150(437)	1174 (590)	77 (85)	<0.001
AUC EAR challenge 2	1370 (533)	932 (376)	134 (155)	<0.001
AUC EAR challenge 3	1530 (792)	1320 (671)	177 (185)	<0.001
p value within group	0.05	0.13	0.25	
AUC LAR challenge 1	5799 (4044)	1823 (1924)	575 (497)	<0.001
AUC LAR challenge 2	6241 (3656)	1519 (1230)	657 (787)	<0.001
AUC LAR challenge 3	6601 (3563)	1837 (1039)	743 (700)	<0.001
p value within group	0.72	0.92	0.61	

Values are means with SD in parentheses. EAR = Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). AUC area under the curve. p values are calculated using Kruskal Wallis test (between groups) or Friedmans' test (within groups).

Table 4-10 Mean maximal changes in FEV₁ following repeated inhalation challenges with allergen and methacholine in subjects who completed repeated bronchoscopy.

	Allergen (n=12)	Methacholine (n=12)	p value
FEV ₁ EAR challenge 1	-20.8 (6.3)	-22.1 (7.5)	0.51
FEV ₁ EAR challenge 2	-22.3 (7.6)	-23.1 (8.3)	0.98
FEV ₁ EAR challenge 3	-20.5 (9.1)	-22.1 (6.9)	0.67
p value within group	0.92	0.89	
FEV ₁ LAR challenge 1	-23.1 (12.4)	-8.5 (7.0)	0.001
FEV ₁ LAR challenge 2	-25.4 (15.2)	-5.7 (3.8)	<0.001
FEV ₁ LAR challenge 3	-27.5 (14.4)	-9.7 (8.8)	0.001
p value within group	0.17	0.54	

EAR = lowest FEV₁ measured during Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). p values are calculated using Mann-Whitney Test between groups and Friedman's Test within groups.

Table 4-11 Area under the curve for changes in FEV₁ following repeated inhalation challenges with allergen and methacholine in subjects who completed repeated bronchoscopy.

	Allergen (n=12)	Methacholine (n=12)	p value
AUC EAR challenge 1	1150(437)	1174 (590)	0.84
AUC EAR challenge 2	1370 (533)	932 (376)	0.03
AUC EAR challenge 3	1530 (792)	1320 (671)	0.55
p value within group	0.05	0.13	
AUC LAR challenge 1	5799 (4044)	1823 (1924)	<0.001
AUC LAR challenge 2	6241 (3656)	1519 (1230)	0.001
AUC LAR challenge 3	6601 (3563)	1837 (1039)	<0.001
p value within group	0.72	0.92	

Values are means with SD in parentheses. EAR = Early asthmatic reaction (0 to 120 mins), LAR = Late asthmatic reaction (150 to 600 mins). AUC area under the curve. p values are calculated using Mann-Whitney Test (between groups) or Friedmans' test (within groups).

4.3.4 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed during bronchoscopy as previously described. The amount of lavage fluid retrieved from the volunteers was similar between the four groups at each bronchoscopy (Table 4-12). The cellularity of the bronchoalveolar lavage fluid was similar between the four groups prior to inhalational challenge, with the allergen challenge group BAL containing median (IQR) $7.6 (6.7 - 11.7) \times 10^6$ cells, the methacholine group $6.0 (4.2 - 9.7)$, the saline group $8.9 (5.4 - 11.3)$ and the salbutamol / methacholine group $8.2 (5.8 - 11.0)$ ($p=0.54$). There was no significant within or between group differences for the total cell count after the inhalation challenges (Table 4-13). If, rather than examining total cell count, the concentration of cells per ml of recovered BAL fluid is assessed, this too was similar prior to challenge, with the allergen challenge group having median (IQR) $14.7 (13.2 - 23.5) \times 10^6$ cells per 100ml of recovered BAL fluid, the methacholine group $15.4 (10.6 - 26.3)$, the saline group $21.7 (14.9 - 31.4)$ and the salbutamol / methacholine group $19.9 (13.9 - 23.4)$ ($p=0.53$). Following inhalation challenge there was an increase in the number of cells per ml of BAL fluid recovered in the allergen group to median (IQR) of $23.4 (17.0 - 38.6) \times 10^6$ cells per 100ml of recovered BAL ($p=0.02$ for within group difference), though the change in the concentration of cells in the recovered BAL was not different between the four challenge groups ($p=0.29$) (Table 4-14).

Using cytospins made from the BAL fluid as described in Chapter 2, the numbers of eosinophils, lymphocytes, macrophages and epithelial cells were counted and expressed as a percentage of the total 400 cells counted in each instance. Prior to airway challenges there were no significant differences in any of the cell counts; Median (IQR) pre challenge percentage eosinophils in the allergen group was 1.25% ($1.00 - 5.75$), in the methacholine group 1.25 ($0.81 - 2.25$), in the saline group 0.75 ($0.25 - 1.94$) and in the salbutamol / methacholine group 1.75 ($0.31 - 6.00$) ($p=0.50$ for between group difference). Following repeated airway challenges there was a significant increase in the

percentage of eosinophils in the allergen group to median (IQR) percentage of 4.13% (2.44 - 12.88) ($p=0.01$ for within group change), whilst there was no significant difference in the other three challenge groups (Table 4-15). When the change in eosinophil count was calculated between the pre and post challenge bronchoscopies the median (IQR) change in the allergen group was 3.13 (1.06 - 10.13), in the methacholine group -0.25 (-1.09 - 0.50), in the saline group -0.25 (-1.44 - 0.19) and in the salbutamol group -0.25 (-1.38 - 0.19). The between group difference was significant between the allergen and all other groups ($p=0.004$). The results are also shown in Figure 4-1.

The percentage of all other cells in the BAL (macrophages, neutrophils, lymphocytes and epithelial cells) was not significantly different prior to or following repeated inhaled challenges between any of the groups as can be seen in Table 4-16, Table 4-17, Table 4-18 and Table 4-19.

Table 4-12 Percentage of bronchoalveolar lavage fluid recovered at bronchoscopy before and after repeated inhaled challenges.

	Percentage of bronchoalveolar lavage fluid recovered at bronchoscopy			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	46.4 (39.5 - 62.0)	43.8 (32.0 - 55.5)	0.35	-7.8 (-21.8 - 14.9)
Methacholine	44.3 (35.0 - 57.5)	38.5 (34.1 - 50.9)	0.29	-6.0 (-14.3 - 6.6)
Saline	46.3 (38.8 - 55.0)	45.6 (36.6 - 52.5)	0.64	-2.5 (-12.5 - 12.2)
Salbutamol / methacholine	49.0 (41.3 - 57.8)	51.8 (41.3 - 69.7)	0.37	5.6 (-11.0 - 19.3)
p value	0.77	0.12		0.53

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Table 4-13 Total cells recovered in bronchoalveolar lavage before and after repeated inhaled challenges.

	Total cells recovered at bronchoscopy ($\times 10^6$ cells)			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	7.6 (6.7 - 11.7)	8.8 (5.1 - 17.7)	0.58	-0.25 (-4.1 - 6.9)
Methacholine	6.0 (4.2 - 9.7)	7.2 (4.9 - 11.4)	0.43	1.2 (-2.4 - 4.2)
Saline	8.9 (5.4 - 11.3)	7.5 (6.2 - 10.5)	0.94	-0.7 (-2.6 - 3.8)
Salbutamol / methacholine	8.2 (5.8 - 11.0)	8.3 (6.3 - 12.3)	0.93	0.1 (-2.9 - 3.2)
p value	0.54	0.89		0.92

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Table 4-14 Total cells recovered at bronchoscopy per 100ml of recovered BAL fluid before and after repeated inhaled challenges.

Total cells recovered at bronchoscopy per ml BAL fluid ($\times 10^6$ cells per 100 ml BAL fluid)				
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	14.7 (13.2 - 23.5)	23.4 (17.0 - 38.6)	0.02	8.3 (-1.8 - 16.6)
Methacholine	15.4 (10.6 - 26.3)	20.4 (12.3 - 27.4)	0.64	-1.0 (-5.6 - 8.8)
Saline	21.7 (14.9 - 31.4)	20.6 (14.8 - 29.3)	0.75	0.2 (-11.0 - 15.9)
Salbutamol / methacholine	19.9 (13.9 - 23.4)	18.9 (13.6 - 23.5)	0.70	-2.2 (-4.5 - 3.9)
p value	0.53	0.60		0.29

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Table 4-15 Percentage of eosinophils in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.

	BAL percentage eosinophils			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	1.3 (1.0 - 5.8)	4.1 (2.4 - 12.9)	0.01	3.1 (1.1 - 10.1)
Methacholine	1.3 (0.8 - 2.3)	1.0 (0.6 - 1.5)	0.47	-0.3 (-1.1 - 0.5)
Saline	0.8 (0.3 - 1.9)	0.4 (0.1 - 1.1)	0.10	-0.3 (-1.4 - 0.2)
Salbutamol / methacholine	1.8 (0.3 - 6.0)	2.0 (0.8 - 6.2)	0.26	-0.3 (-1.4 - 0.2)
p value	0.50	<0.001		0.004

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage

Figure 4-1 Eosinophils as a percentage of total cells recovered at bronchoalveolar lavage following repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine, C - salbutamol followed by methacholine and D - saline. Change in percentage eosinophil count is shown in panel E. Bars show mean.

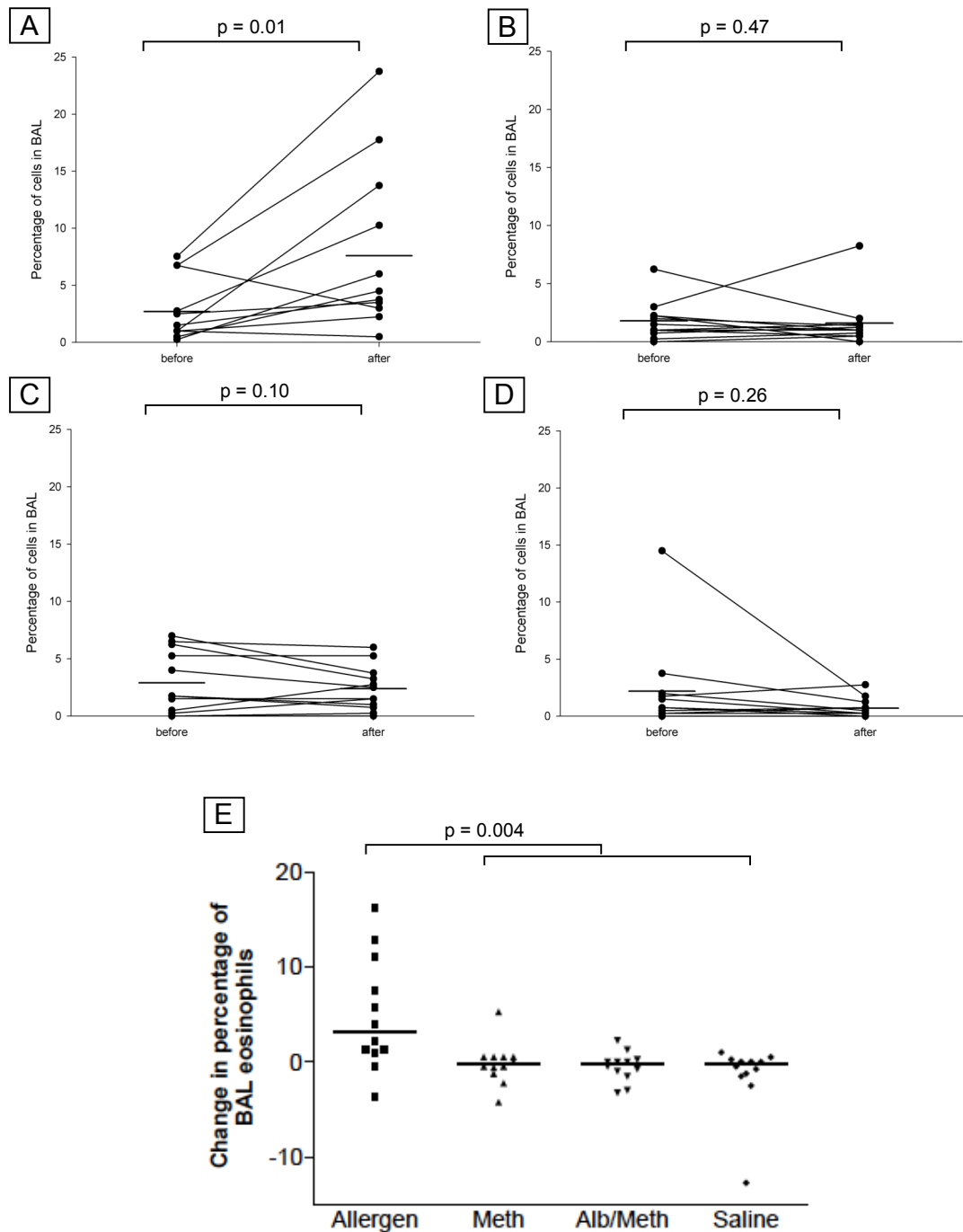


Table 4-16 Percentage of neutrophils in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.

	BAL percentage neutrophils			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	3.4 (1.1 - 4.9)	3.5 (1.5 - 7.6)	0.64	0.5 (-0.2 - 3.1)
Methacholine	2.3 (1.4 - 5.7)	2.4 (1.2 - 8.1)	0.91	0.00 (-2.6 - 2.0)
Saline	2.0 (1.1 - 2.5)	2.4 (2.0 - 4.4)	0.41	0.63 (-0.2 - 2.4)
Salbutamol / methacholine	1.9 (1.1 - 4.1)	4.9 (2.3 - 8.9)	0.05	2.1 (0.4 - 5.7)
p value	0.54	0.44		0.21

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage

Table 4-17 Percentage of macrophages in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.

	BAL percentage of macrophages			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	85.4 (79.3 - 88.7)	74.8 (55.9 - 88.9)	0.07	-10.1 (-22.0 - 1.4)
Methacholine	83.4 (72.7 - 89.9)	85.4 (71.5 - 90.9)	0.70	1.1 (-5.0 - 7.2)
Saline	90.0 (85.1 - 94.4)	87.1 (83.8 - 91.5)	0.94	0.6 (-9.6 - 5.6)
Salbutamol / methacholine	87.1 (82.6 - 92.8)	86.5 (83.8 - 91.4)	0.42	-0.8 (-5.3 - 1.2)
p value	0.14	0.13		0.15

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage

Table 4-18 Percentage of lymphocytes in cells recovered in bronchoalveolar lavage before and after repeated airway challenges.

	BAL percentage of lymphocytes			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	1.3 (0.8 - 1.7)	0.8 (0.6 - 1.0)	0.12	-0.1 (-0.9 - 0.0)
Methacholine	1.3 (1.0 - 2.2)	0.6 (0.5 - 1.4)	0.11	-0.5 (1.6 - 0.1)
Saline	1.1 (0.5 - 1.3)	1.2 (0.5 - 1.3)	0.78	0.0 (0.3 - 0.4)
Salbutamol / methacholine	1.0 (0.8 - 1.5)	1.0 (0.8 - 1.8)	0.52	0.00 (-0.4 - 0.5)
p value	0.39	0.47		0.13

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage

Table 4-19 Percentage of epithelial cells as percentage of total cells recovered in bronchoalveolar lavage before and after repeated airway challenges.

	BAL percentage of epithelial cells			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	6.5 (4.8 - 11.9)	9.9 (3.9 - 18.0)	0.14	3.3 (-2.5 - 7.4)
Methacholine	10.6 (6.7 - 17.2)	9.4 (6.7 - 17.2)	0.81	0.4 (-8.3 - 5.7)
Saline	6.5 (3.1 - 8.9)	7.9 (5.0 - 10.2)	0.41	1.3 (-3.3 - 6.6)
Salbutamol / methacholine	4.8 (3.1 - 8.1)	3.6 (1.8 - 4.7)	0.76	0.1 (-3.2 - 1.2)
p value	0.09	0.03		0.42

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage

4.3.5 BAL ELISA measurements of inflammation

BAL interleukin 8 (IL8) and eosinophil cationic protein (ECP) were measured in unconcentrated BAL in order to give a measure of inflammation within the airway. ELISA assays were performed according to manufacturers instructions as previously described.

BAL ECP concentrations were not significantly different between the four groups prior to repeated inhaled challenge, with the median (IQR) concentration in ng/ml in the allergen group of 1.5 (0.5 - 4.5) ng/ml, in the methacholine group 0.8 (0.3 - 3.7), in the saline group 0.4 (0.1 - 3.2) and in the salbutamol / methacholine group 2.1 (1.3 - 3.9) ($p=0.09$ for between group difference). Following repeated inhalation challenge there was a significant within group increase in ECP concentration in the allergen challenge group, the ECP concentration rising to 11.9 (6.0 - 35.2) ng/ml ($p=0.002$) whilst there was no significant increase in any of the other exposure groups (Table 4-20). When the difference between post exposure and pre exposure ECP concentration in the BAL is calculated it was 9.1 (3.4 - 31.5) in the allergen group, 0.5 (-0.3 - 3.1) in the methacholine group, -0.2 (-1.5 - 0.6) in the saline group and 1.5 (-1.4 - 3.6) in the salbutamol / methacholine group ($p=0.001$ for between group differences). These data are also shown in Figure 4-2.

Interleukin 8 concentrations (pg/ml) were not significantly different between the four groups prior to inhalation challenge; the median (IQR) concentrations were 19.7 (12.8 - 23.6) pg/ml in the allergen group, 23.0 (20.4 - 28.8) in the methacholine group, 25.0 (18.0 - 45.1) in the saline group and 21.8 (16.0 - 25.9) in the salbutamol / methacholine group ($p=0.15$ for between group difference) as shown in Table 4-21. Following inhalation challenges there was a significant within group increase only within the allergen group, the median (IQR) concentration increasing to 49.0 (33.7 - 75.3) ($p=0.006$ for within group

change). There were no other significant increases within the other three groups as shown in Table 4-21.

Table 4-20 Concentration of eosinophil cationic protein in BAL fluid before and after repeated inhaled challenges.

	BAL concentration of ECP (ng/ml)		p value within group	Change (Δ) between challenges
	Pre challenge	Post challenge		
Allergen	1.5 (0.5 - 4.5)	11.9 (6.0 - 35.2)	0.002	9.1 (3.4 - 31.5)
Methacholine	0.8 (0.3 - 3.7)	2.8 (1.0 - 5.5)	0.14	0.5 (-0.3 - 3.1)
Saline	0.4 (0.1 - 3.2)	0.6 (0.2 - 3.5)	0.58	-0.2 (-1.5 - 0.6)
Salbutamol / methacholine	2.1 (1.3 - 3.9)	4.9 (3.2 - 5.9)	0.35	1.5 (-1.4 - 3.6)
p value	0.204	0.001		0.001

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, ECP - eosinophil cationic protein

Figure 4-2 Eosinophil cationic protein (ECP) measured in bronchoalveolar lavage fluid following repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine, C - salbutamol followed by methacholine and D - saline. Change in ECP concentration is shown in panel E. Bars show mean.

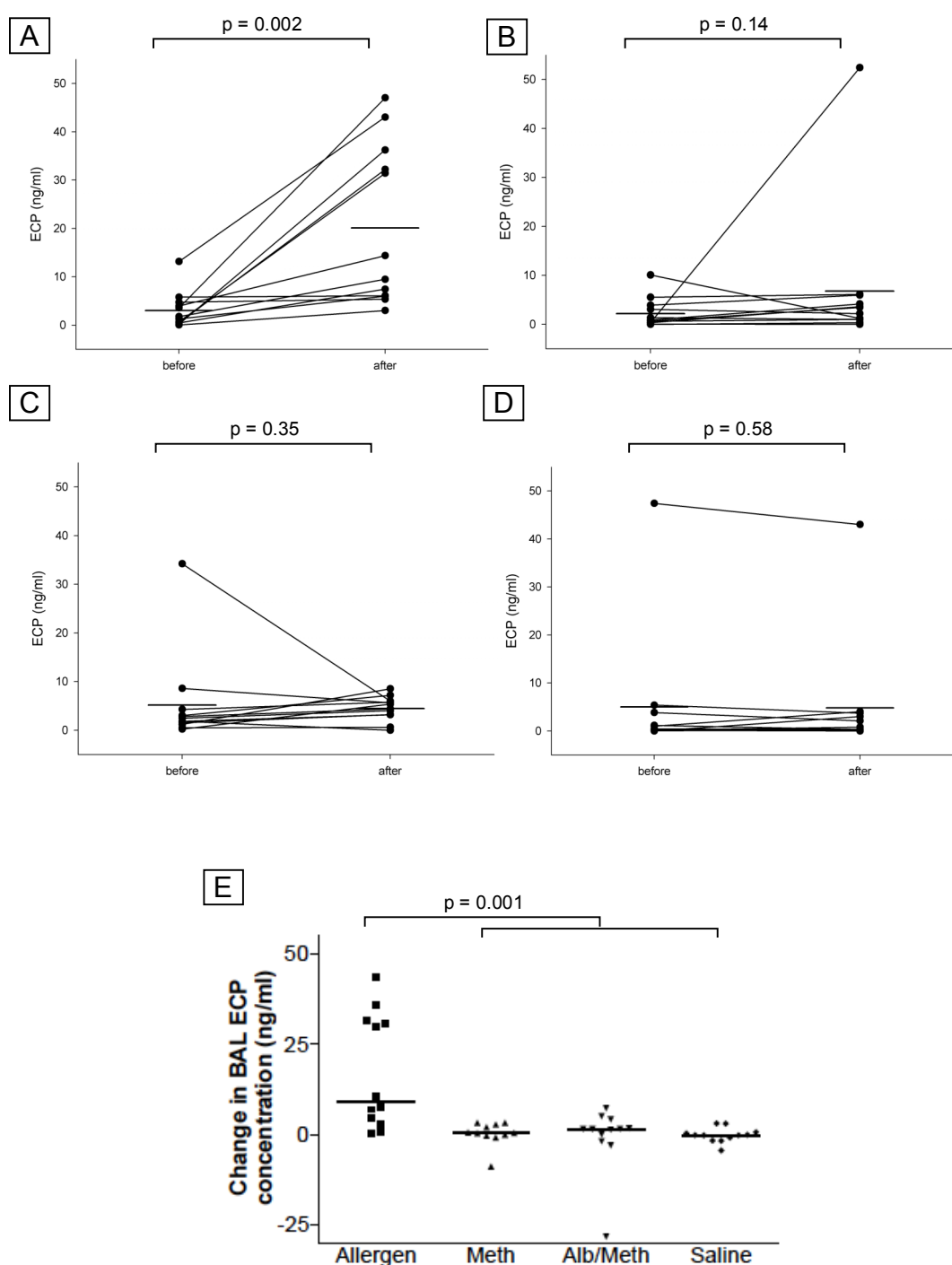


Table 4-21 Concentration of Interleukin 8 in BAL fluid before and after repeated inhaled challenges.

	BAL concentration of IL8 (pg/ml)			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	19.7 (12.8 - 23.6)	49.0 (33.7 - 75.3)	0.006	31.6 (3.5 - 61.0)
Methacholine	23.0 (20.4 - 28.8)	29.5 (19.1 - 40.7)	0.388	2.9 (-5.5 - 13.8)
Saline	25.0 (18.0 - 45.1)	32.1 (25.7 - 65.9)	0.530	2.3 (-10.8 - 30.7)
Salbutamol / methacholine	21.8 (16.0 - 25.9)	21.1 (13.3 - 40.9)	0.583	0.2 (-5.9 - 18.4)
p value	0.15	0.16		0.12

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, IL8 - interleukin 8.

4.3.6 Immunohistochemistry

Immunohistochemistry was performed as previously described using antibodies detailed in Chapter 2. Cell counts in the submucosa (cells per mm² of submucosa) and the epithelium (cells per mm of epithelial length) were measured using immunohistochemistry for CD68 positive macrophages, AA1 positive mast cells and EG2 positive eosinophils. The method for calculating the area of the submucosa of interest and the length of the epithelium are detailed in Chapter 2. Typical staining of eosinophils, macrophages and mast cells are shown in Figure 4-3, Figure 4-4 and Figure 4-5 respectively.

Figure 4-3 Immunohistochemical staining of eosinophils with anti EG2 antibody (arrow). Chromogen AEC. Bar = 100µm.

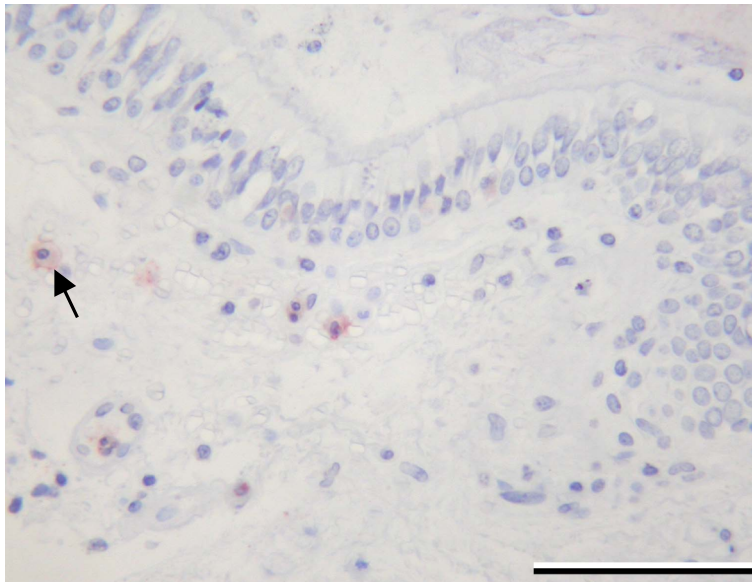


Figure 4-4 Immunohistochemical staining of macrophages using an anti CD68 antibody (arrow). Chromogen AEC. Bar = 100 μ m.

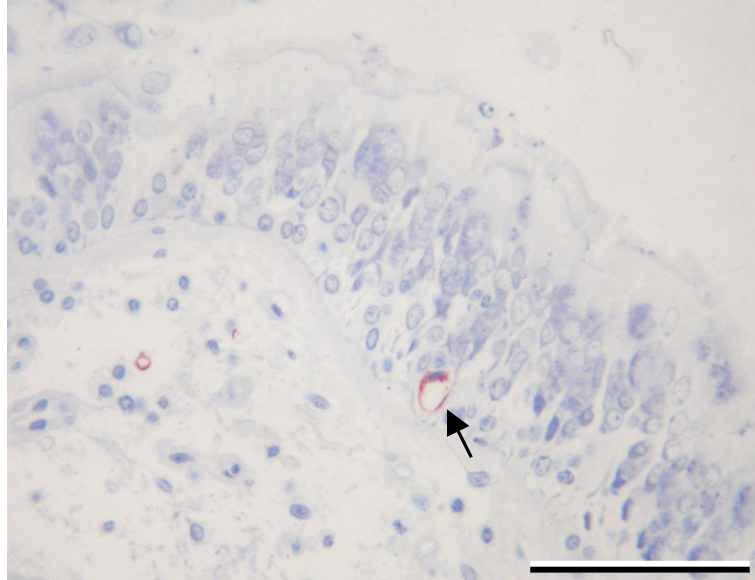
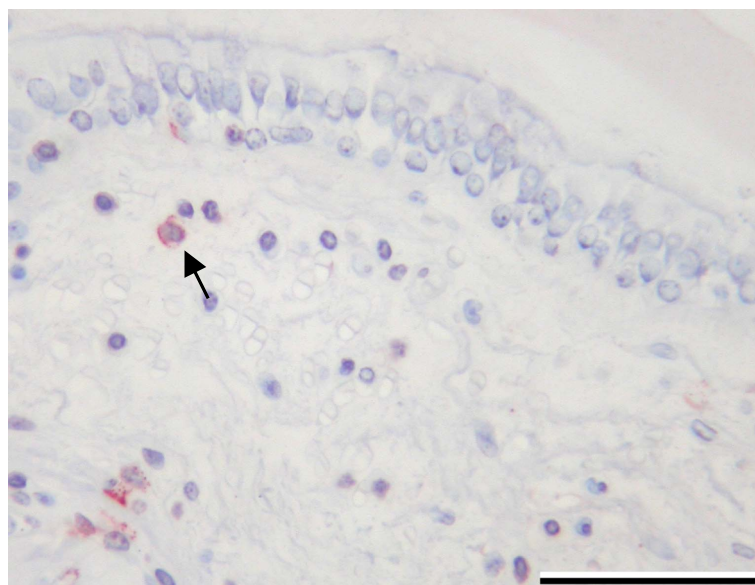


Figure 4-5 Immunohistochemical staining of mast cells using an anti AA1 antibody (arrow). Chromogen AEC. Bar = 100 μ m.



4.3.7 Epithelial cells

Very few eosinophils or macrophages were found within the epithelium either before or after repeated inhaled challenges; there were no between group differences for either cell type in the epithelium prior to or after any challenge as can be seen in Table 4-22 and Table 4-23. There were more mast cells detected in the epithelium than eosinophils or macrophages, though there were still no significant between group differences before or after repeated inhalation challenge as is shown in Table 4-24.

Table 4-22 Eosinophils per millimetre of bronchial mucosa epithelium before and after repeated inhaled challenges.

	Eosinophils per mm epithelium			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.32	0.0 (0.0 - 0.0)
Methacholine	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	1.00	0.0 (0.0 - 0.0)
Saline	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.66	0.0 (0.0 - 0.0)
Salbutamol / methacholine	0.0 (0.0 - 0.4)	0.0 (0.0 - 0.0)	0.18	0.0 (0.0 - 0.0)
p value	0.25	1.00		0.66

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Table 4-23 Macrophages per millimetre of bronchial mucosa epithelium before and after repeated inhaled challenges.

	Macrophages per mm epithelium			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	0.0 (0.0 - 0.3)	0.0 (0.0 - 0.0)	0.10	0.0 (-0.3 - 0.0)
Methacholine	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	1.00	0.0 (0.0 - 0.0)
Saline	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.18	0.0 (0.0 - 0.0)
Salbutamol / methacholine	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.56	0.0 (0.0 - 0.0)
p value	0.57	0.54		0.18

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Table 4-24 Mast cells per millimetre of bronchial mucosa epithelium before and after repeated inhaled challenges.

	Mast cells per mm epithelium			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	0.2 (0.0 - 1.4)	0.4 (0.0 - 1.7)	0.31	0.0 (0.0 - 0.4)
Methacholine	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.2)	0.69	0.0 (0.0 - 0.2)
Saline	0.5 (0.0 - 2.3)	0.0 (0.0 - 1.3)	0.31	0.0 (-1.0 - 0.0)
Salbutamol / methacholine	0.0 (0.0 - 2.4)	0.0 (0.0 - 0.0)	0.17	0.0 (-2.4 - 0.0)
p value	0.58	0.35		0.26

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

4.3.8 Submucosal cells

Tissue eosinophils

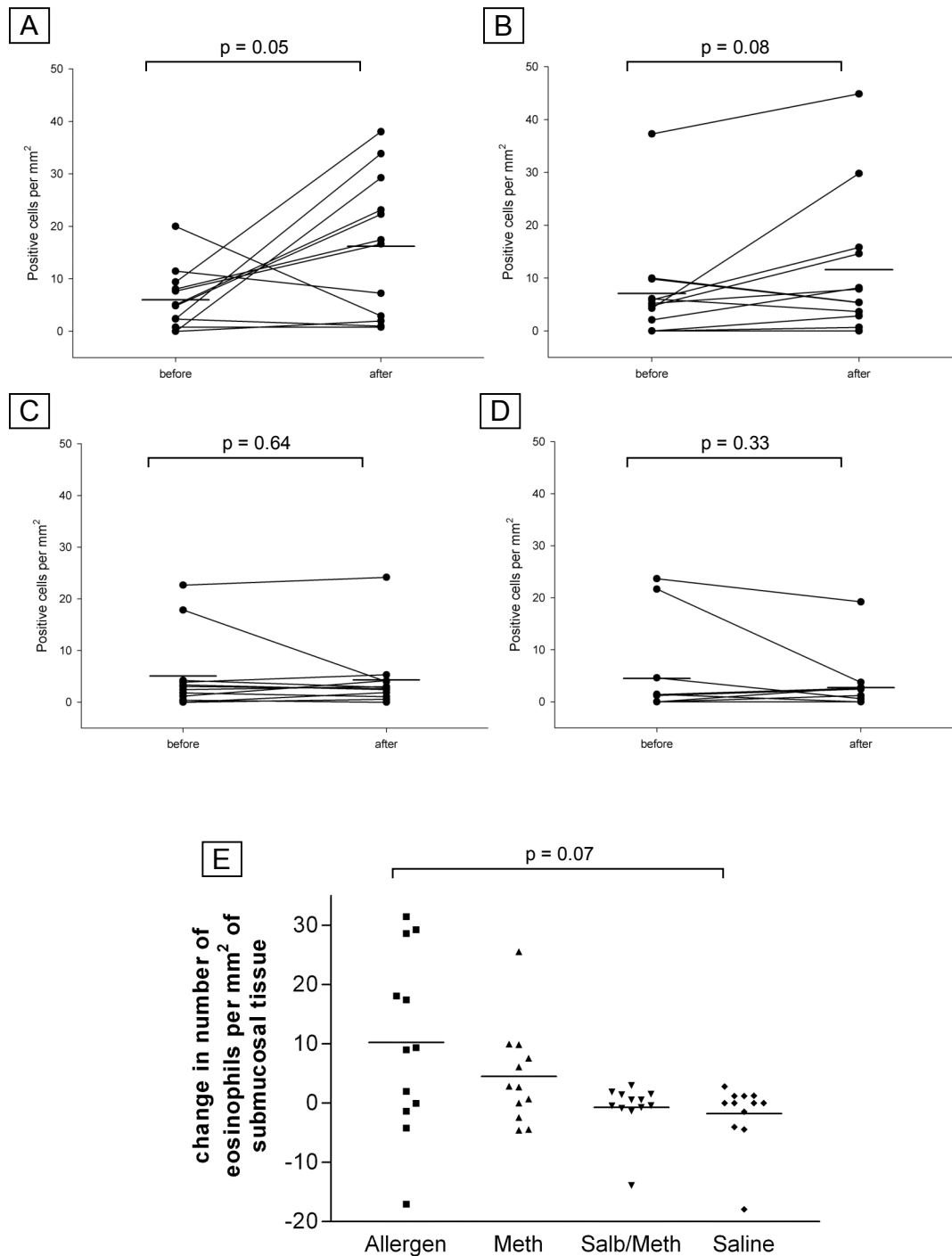
Tissue eosinophils were stained and counted, then expressed as cells per square mm of submucosal tissue. The median (IQR) numbers of cells per mm² prior to challenge in the allergen group were 3.25 (0.63 - 6.63) cells/mm², in the methacholine group 2.75 (0.38 - 6.75), in the saline group 0.75 (0.00 - 2.25) and in the salbutamol / methacholine group 2.75 (0.63 - 5.75) ($p=0.57$). Following inhalation challenges there was an increase in the numbers of eosinophils in the submucosa in the allergen challenge group, post challenge median (IQR) was 11.00 (1.38 - 14.38) ($p=0.05$). There was no increase in any of the other groups. The difference between the pre and post cell counts were 9.16 (-1.02 - 25.99) in the allergen group, 2.78 (-1.82 - 9.30) in the methacholine group, 0.00 (-3.39 - 1.22) in the saline group and 0.07 (-0.85 - 1.50) in the salbutamol / methacholine group. Although the difference appears larger in the allergen group, the between group difference is not statistically significant ($p=0.07$). These data are shown in Table 4-25 and Figure 4-6.

Table 4-25 Eosinophils per square millimetre of bronchial submucosal tissue before and after repeated inhaled challenges.

	Eosinophils per mm ² submucosa			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	3.3 (0.6 - 6.6)	11.0 (1.4 - 14.4)	0.05	9.2 (-1.0 - 26.0)
Methacholine	2.8 (0.4 - 6.8)	4.3 (2.0 - 9.3)	0.08	2.8 (-1.8 - 9.3)
Saline	0.8 (0.00 - 2.3)	0.5 (0.0 - 2.1)	0.33	0.00 (-3.4 - 1.2)
Salbutamol / methacholine	2.8 (0.6 - 5.8)	2.0 (0.6 - 2.9)	0.64	0.07 (-0.9 - 1.5)
p value between groups	0.24	0.001		0.07

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Figure 4-6 Eosinophils per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine, C - salbutamol followed by methacholine and D - saline. Change in number of eosinophils per square mm shown in panel E. Bars show mean.



Tissue mast cells and macrophages

Mast cells numbers in the submucosa in the four exposure groups prior to challenge were 48.17 (20.39 - 66.74) cells / mm² in the allergen group, 23.12 (16.67 - 53.78) in the methacholine group, 22.44 (16.28 - 30.39) in the saline group and 16.42 (4.89 - 36.37) in the salbutamol / methacholine group (p=0.15). Following inhalation challenges there was a significant increase in the number of mast cells in the methacholine group, with the median (IQR) number of cells increasing to 37.08 (20.09 - 94.03) (p=0.03 within group) whilst there were no significant changes in the other challenge groups. Calculation of the difference showed a median (IQR) difference of 5.85 (-30.45 - 14.48) in the allergen group, 15.17 (-1.63 - 33.11) in the methacholine group, 3.51 (-12.20 - 8.61) in the saline group and -3.05 (-25.64 - 0.46) in the salbutamol / methacholine group. Although the increase in the methacholine group was greater than the other groups, there was no statistically significant difference when comparing between the four groups (p=0.10). If the change in mast cells per mm² of submucosa is compared between the methacholine group and the salbutamol methacholine group, there was a significant difference (p=0.03). These data are shown in Table 4-26 and in Figure 4-7.

The macrophage numbers in the submucosal tissue of the endobronchial biopsies were similar in all four groups prior to inhalation challenge, and did not change significantly following as shown in Table 4-27.

Table 4-26 Mast cells per square millimetre of bronchial submucosal tissue before and after repeated inhaled challenges.

	Mast cells per mm ² of submucosa			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	48.2 (20.4 - 66.7)	37.8 (20.2 - 71.0)	0.88	5.9 (-30.5 - 14.5)
Methacholine	23.1 (16.7 - 53.8)	37.1 (20.1 - 94.0)	0.03	15.2 (-1.6 - 33.1)
Saline	22.4 (16.3 - 30.4)	19.6 (12.2 - 31.9)	0.88	3.5 (-12.2 - 8.6)
Salbutamol / methacholine	16.4 (4.9 - 36.4)	7.6 (1.4 - 17.1)	0.25	-3.1 (-25.6 - 0.5)
p value between groups	0.15	0.003		0.10

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. Comparison of methacholine change in mast cells per square mm of submusoca to that induced by salbutamol / methacholine showed a significant difference (p=0.03), neither saline (p=0.052) nor allergen (p=0.29) were significantly different (Mann-Whitney Test).

Figure 4-7 Mast cells per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine, C - salbutamol followed by methacholine and D - saline. Change in number of mast cells per square mm shown in panel E. Bars show mean.

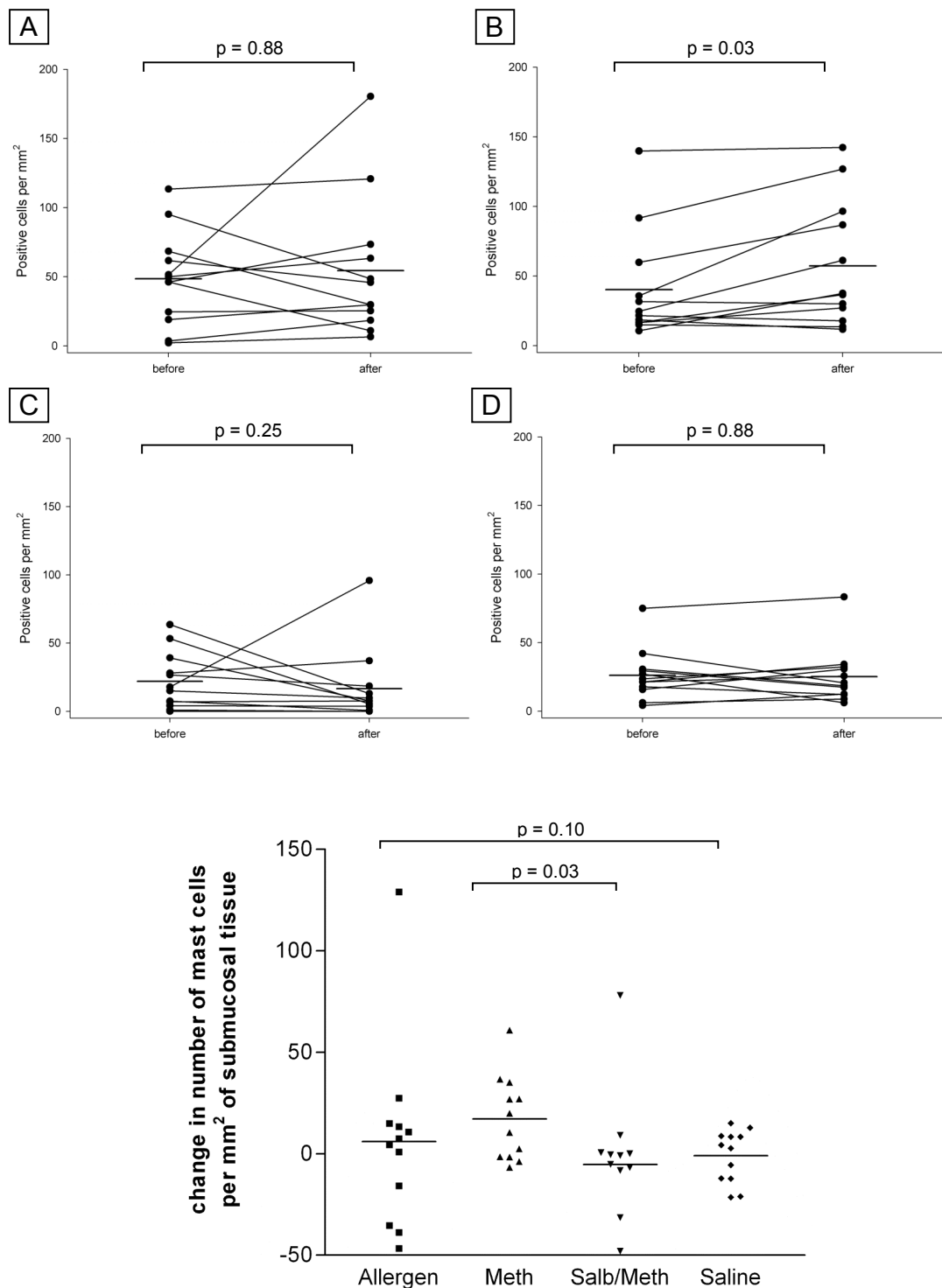


Table 4-27 Macrophages per square millimetre of bronchial submucosal tissue before and after repeated inhaled challenges.

	Macrophages per mm ² submucosa			
	Pre challenge	Post challenge	p value within group	Change (Δ) between challenges
Allergen	1.9 (0.0 - 6.4)	6.1 (0.6 - 14.0)	0.10	1.7 (0.0 - 12.4)
Methacholine	1.6 (0.2 - 3.6)	2.6 (1.1 - 9.1)	0.06	2.0 (-0.3 - 7.0)
Saline	0.3 (0.0 - 4.4)	0.0 (0.0 - 4.0)	0.57	0.0 (-2.0 - 0.6)
Salbutamol / methacholine	1.0 (0.1 - 4.5)	3.2 (0.7 - 11.2)	0.41	2.0 (0.2 - 3.5)
p value between groups	0.83	0.11		0.09

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

4.4 Discussion

Our results show a significant increase in eosinophilic inflammation in repeatedly allergen challenged individuals compared to individuals exposed repeatedly to inhaled methacholine, saline or salbutamol followed by methacholine. In addition the data also show an increase in mast cells within the tissue in the methacholine challenged group when considered as a paired change before and after repeated challenge, and also when compared to the best control group - that of salbutamol followed by methacholine.

4.4.1 Repeated bronchoscopy

The second bronchoscopies in the allergen group were more difficult to perform due to subjective difficulty in the ability to anaesthetise the upper airway using spray and gel lignocaine, this subjective difficulty is mirrored in a significantly larger number of volunteers failing to complete the second bronchoscopy in the allergen group compared to the other three challenge groups. Bronchoscopy following repeated inhalation allergen challenge has not been performed previously, though repeated research bronchoscopies have. Kariyawasam *et al* reported their experiences of performing three consecutive research bronchoscopies prior to and 24 hours and seven days after single inhalation allergen challenge (Kariyawasam 2007). In this situation there were no significant adverse effects and no apparent difficulties in inducing upper airway anaesthesia. It is well described that both bronchial allergen challenge and nasal allergen challenge induce inflammation (Kämpe 2010, van Rensen 2009, Gauvreau 2000) and also that the clinical analgesic effects of local anaesthetics are influenced by inflammation in the tissues; this is especially well reported in the dental literature (Potocnik 1999). It has been thought that the inflamed tissue produces acidic products which result in ionisation of the local anaesthetic molecules; these ionised molecules are then not able to penetrate the nerve fibre membranes and there block fast voltage gated sodium channels,

and therefore prevent nerve conduction. This mechanism does not however hold up to examination, as most local anaesthetic preparations are acidic in nature (approximately pH 4) and lowering of the tissue pH in an experimental system to pH 6.9 resulted in the same membrane penetration of lignocaine as that found with a tissue pH of 7.4 (Tsuchiya 2007). The authors of this study propose an alternative mechanism by which the clinical findings of decreased anaesthetic activity may be caused; that of increased blood flow and increased vascular permeability in an area of inflammation. Such increased blood flow would clear the drug more quickly, whilst the increased vascular permeability results in increased plasma proteins in the pericellular space; these anaesthetic compounds are highly plasma protein bound and inactive once bound. Whatever the mechanism, the reduction in the ability to anaesthetise the upper airway led, subjectively at least, to the reduction in the numbers of subjects undergoing allergen challenge completing repeated bronchoscopy.

As stated above, there were no differences in the baseline characteristics, falls in FEV₁, symptoms or reliever medication use which would suggest no increased effects of the allergen challenge in these subjects (though the numbers were small). There was however no measure of inflammation, which may have been greater in this subgroup, which may have in turn made upper airway anaesthesia more difficult. It would have been useful to have a non invasive measure of airway inflammation, such as that from exhaled nitric oxide (eNO), or induced sputum to see if the levels of inflammation did correlate with the inability to obtain repeated bronchoscopy. Induced sputum was not performed as it may have confounded the results obtained, however not performing eNO measurement was an unfortunate omission, which should be corrected should this or similar studies be performed again in the future. It is of course possible that there were clinical predictors of which subjects might not tolerate the repeated bronchoscopy after the repeated airway challenges but these were not detected due to the small numbers and subsequent lack of power.

4.4.2 Completed bronchoscopy groups

As not all individuals in the allergen challenge group completed both bronchoscopies, it was important to establish that the differences between the original inhalation challenge groups were maintained once those individuals data was removed. As shown above, the results were not affected by the exclusion of the 4 bronchoscopy failures. The aim of inducing a matched initial fall in FEV₁ between the allergen and methacholine challenges at each of three challenges was achieved. In addition there remained a significant difference in response between the active challenge groups and the saline and salbutamol / methacholine groups.

4.4.3 BAL responses to repeated inhalation challenge

There was no difference in the recovery of BAL fluid before or after inhalation challenge with any of the challenge groups, which suggests that in the allergen challenge group, once the bronchoscope had been passed through the cords and the airway anaesthetised, the bronchoscopy was no more difficult to perform than in the other groups. The total cells recovered in the four groups was not different before or after inhalation challenge, which considering the increase in the percentage of eosinophils was unexpected as more cells were being recruited to the airway lumen, it might have been thought that there would have been an increase in total cell numbers as well as the percentage of eosinophils in the allergen group. When examining concentration of cells per 100ml of recovered BAL fluid, there was indeed a significant increase in the allergen challenge group, but this was only present as a within group difference and was not maintained when the change in cell concentrations between the four groups was considered. This increase may therefore have been real but the small numbers in the study precluded a between group significant change, or the result may be purely due to repeated statistical analysis. Increasing the numbers in the study would answer that question, though would be difficult to achieve.

As expected there was a significant increase in both IL8 and ECP in the allergen challenge group, though only the change in ECP was significantly different from the change in the other groups. There was no increase in the IL8 or ECP levels in the BAL in any other group, which was as expected. Specifically there was no generalised inflammatory response, as measured by the IL8 or eosinophilic inflammatory response (as reflected by the ECP measurements) in the methacholine or control groups, which suggests that bronchoconstriction *per se* does not induce either a generalised inflammatory or eosinophilic inflammatory response.

4.4.4 Tissue responses to repeated inhalation challenge

Eosinophils

The number of eosinophils within the submucosa of the bronchial biopsies increased significantly within the allergen group, though when the change in numbers was compared between the different inhalation challenge groups there was no significant change. This raises the question of why there was a significant increase in both BAL ECP and eosinophil percentages in the allergen group compared to the other groups whilst there was not a concomitant increase between the groups in the tissue eosinophils? Why, if there is an eosinophilic inflammatory response in the allergen challenge group was this reflected in the BAL measures, but not in the tissue measures of inflammation?

Although serial BAL and biopsy measurements of inflammation in humans are difficult, such measures have been performed in animal models. Using Brown Norway rats which had been sensitised to ovalbumin (OVA) it has been shown that after a single OVA challenge there was an increase in tissue eosinophils by 24hrs with a peak at 48hrs post challenge. The peak in BAL fluid eosinophilia however did not occur until 72 hours after challenge (Schneider 1997). Lung eosinophilia persisted for 6 days after the initial challenge with slow resolution.

The sampling in the current study took place 96 hours after the last inhalation challenge. If the timings of peak inflammation can be directly extrapolated from a single OVA challenge in the rat to multiple inhalation challenge in humans (which is unlikely), this would suggest that the samples were taken significantly after the peak of tissue eosinophilia, and shortly after the peak of BAL eosinophilia. In human subjects, following a single allergen challenge, bronchoscopies performed at 3 hours and 24 hours after challenge (on separate challenge occasions) show that tissue eosinophils had increased from baseline measurement by 3 hours and were still elevated at 24 hours. BAL measurements of eosinophils were also elevated at both time points, however at 24 hours there was a strong negative correlation between the number of eosinophils in the lumen and in the submucosa (Aalbers 1993).

If the eosinophils are rapidly accumulating in the submucosa, as is expected from the investigations above, then they must also be cleared from the tissue rapidly in order for our data to show no significant difference in tissue eosinophils between the four groups. The absolute number of tissue eosinophils must be a balance between the number entering and the number being cleared from any area. It has been thought in recent years, mostly based on *in vitro* data, that eosinophil clearance from tissues is due to apoptosis and subsequent macrophage phagocytosis (Haslett 1999, Vignola 2000, Walsh 2008). However, our data are more in keeping with eosinophils which have entered the tissue being cleared from the submucosa by egress into the lumen, where they may be measured in the BAL fluid. There is growing evidence that this process is the more likely one to underlie the resolution of airway inflammation *in vivo* (Uller 2006a, Persson 2010).

In animal models, there is little if any evidence of eosinophil apoptosis, even in the case of treatment with steroids, which clear tissue eosinophils more quickly than placebo. Using a rat model where eosinophilic inflammation was induced using tracheal administration of Sephadex beads, there was no evidence of

eosinophil apoptosis using gold standard transmission electron microscopy techniques to identify apoptotic cells (Uller 2001). Steroids administered in this model reduced tissue eosinophilia more rapidly than placebo, whilst having no impact on the number of apoptotic eosinophils; there were however many apoptotic eosinophils seen in the airway lumen. In addition to the absence of eosinophilic apoptosis, no macrophage phagocytosis of eosinophils was seen. This study suggests that the eosinophils are not cleared by apoptosis, rather the authors suggest that the cells are moved from the tissue to the lumen where they are either degraded or cleared by the mucociliary escalator, however there was no data in this study to specifically support this alternative hypothesis (Uller 2001).

In a guinea pig model, where OVA challenge does not rapidly induce eosinophil recruitment, it has been shown that allergen challenge actually rapidly reduces the numbers of tissue eosinophils from 10 minutes after challenge. This reduction is not associated with eosinophil apoptosis, rather with a rapid transit of eosinophils across an undamaged epithelial surface. The authors calculate that in 10 minutes 3.5×10^5 eosinophils leave every cm^2 of epithelium, as concurrent with the reduction in tissue eosinophils there was an increase in luminal eosinophils (Erjefält 2004). Confirmation of these findings using a mouse model with repeated OVA challenge showed that after 2 daily challenges there was a significant increase in tissue but not BAL eosinophils, but after 7 daily challenges there was an increase in both. Interestingly if corticosteroids were administered with the OVA challenges, then egress of eosinophils into the airway lumen was greater (Uller 2006b).

There is also evidence that eosinophil egress into the airway lumen occurs in humans, though using a nasal allergen challenge rather than a bronchial challenge. In this study the authors demonstrate a gradient of eosinophils from the submucosa into the epithelial surface following allergen challenge, again in the absence of apoptotic eosinophils (Uller 2010).

How might such egression be regulated? It has been shown that matrix metalloproteinase 2 (MMP2) reduces tissue inflammation by allowing the egress of inflammatory cells into the bronchial lumen. By generating an MMP2 -/- knockout mouse which is then allergen challenged, it can be shown that the animals have reduced BAL eosinophils, but accumulate these cells in the tissues (Corry 2002).

Our data correlate well with the data from the animal studies, and although we did not observe any significant numbers of epithelial eosinophils following any challenge, the greater increase in the number of BAL eosinophils compared to the tissue eosinophils would suggest that the cells are leaving the bronchial tissue into the lumen, rather than undergoing apoptosis within the tissue. To assess this further, the biopsies from this study could be examined to calculate the proportion of eosinophils detected which were apoptotic, though previous work suggests that this may be futile, with almost no apoptotic eosinophils being detected (Erjefält 2004, Uller 2006b, Uller 2010). It might be possible using an animal model to label bone marrow eosinophil precursors, induce airway inflammation using allergen challenge, and then repeatedly assess the airway inflammatory state by bronchoscopy to assess cellular entry into, then out of the tissue by whichever mechanism is predominant.

Mast cells

Although there was no significant difference in the change in mast cells per mm² of submucosa between the four challenge groups, there was a significant within group difference in the methacholine challenge group and also a difference when compared to the most relevant control for that group, the salbutamol and methacholine group. Although these 'significant' results may be due to repeated statistical analysis, the results are interesting as they suggest that physical contortion of the airway may lead to accumulation of mast cells within the tissue. Experiments on mast cell lines have shown that they are responsive to rotatory shear stress *in vitro* via a process mediated by transient

receptor potential vanilloid protein, in which an influx of extracellular calcium into the mast cells causes cellular degranulation with histamine release (Yang 2009). It is also known from work on the guts of animals, that physical handling of the gut (as occurs during surgery) results in the degranulation of mast cells, which then increases the chances of the formation of a paralytic ileus (The 2008). Also in animals, using limb loading and unloading experiments, it has been demonstrated that the mechanical stress associated with loading and unloading the limbs of rats induced mast cell degranulation (Dumont 2007).

Therefore it is possible that the mechanical stimulation of the airway wall lead to mast cell chemotaxis. Bronchoalveolar lavage fluid from various patient groups has been shown to be chemotactic to mast cells, including from patients with eosinophilic bronchitis (Woodman 2006) and allergic asthma (Olsson 2000a). In the patients with eosinophilic bronchitis the BAL fluid was chemotactic to a human mast cell line (HMC-1) and could be reduced using antibodies to the CXCR1 and CXCR3 receptors; in addition mast cell migration was correlated with levels of both IL8 and Interferon gamma-induced protein 10 kDa (IP-10) (also called CXCL10). In our study the levels of IL8 were not significantly different between groups, though the IP-10 concentration in the BAL was not measured. Interestingly in the study of eosinophilic bronchitis, BAL from patients with asthma was not chemotactic to mast cells, unlike that in other studies (Olsson 2000a). BAL from allergic asthma patients has been shown to be chemotactic to mast cells, and that chemotaxis increased during the pollen season and could be attenuated using blocking antibodies to stem cell factor (SCF) and TGF β , and also by directly blocking G_i protein coupled receptors (Olsson 2000). Examination of the TGF β isoforms showed that concentrations as low as 40 fmol/ml were sufficient to induce chemotaxis, and that all of the TGF β isoforms were capable of attracting mast cells, though TGF β 3 was the most efficient (Olsson 2000b). *In vitro* work by the same group has demonstrated that tumour necrosis factor alpha (TNF α) and interleukins 4 and 8 are also potent mast cell chemottractants (Olsson 2004). It may be that mast

cells from different areas within the airway are responsive to different chemotactic agents; mast cells in smooth muscle in asthmatic airways are known to express more CXCR3 receptor than those found in submucosa. In addition a ligand for CXCR3 (IP-10) has been shown to be secreted more by asthmatic smooth muscle cells *in vitro* than by cells from normal subjects (Brightling 2005).

Therefore it has been shown in the literature that mast cells are responsive to mechanical stress and that they are also subject to a variety of chemotactic agents that are known to be increased in the airways of asthmatic subjects. In order to investigate whether the accumulation of the mast cells in the biopsies of the methacholine group in our study was a real finding, other than performing the study on more individuals, the BAL could be assessed for its capacity to chemottract mast cells *in vitro*, comparing it to the BAL recovered from volunteers in the salbutamol / methacholine challenge group. The other option would be to investigate, perhaps using a multiplex ELISA technique, various of the potential chemottractants for mast cells, such as IP10, SCF, IL4 and TGF β , though the BAL would require concentrating to measure TGF β , and the chemotactic threshold for TGF β has been shown to be orders of magnitude less than the minimum detection threshold of ELISA assays (Olsson 2000b). Another option would be to model epithelial compression *in vitro* and then use the supernatant from the cells to see if this was chemotactive to mast cells.

4.5 Summary

Our study gives some important data on the effects of various inhalation challenges on the inflammatory response within the airway. We demonstrate that repeated allergen challenge evokes an eosinophilic inflammatory response that can still be detected within the lumen and in to a lesser extent in the airway 96 hours after the final challenge. In addition there are many important negatives; repeated bronchoscopy with lavage and biopsy and spirometry do

not cause a detectable inflammatory response. Methacholine may induce a mast cell response that appears to be associated with the bronchoconstriction rather than the direct effect of the methacholine.

These results demonstrate that with a matched degree of initial bronchoconstriction the eosinophilic inflammatory response in the allergen group can be dissociated from the bronchoconstriction alone in the methacholine group. We can therefore use this dissociation to examine the relative effects of bronchoconstriction with and without eosinophilic inflammation on the remodelling of the airway.

Chapter 5 Remodelling responses to repeated inhalation challenges

In the preceding chapters we demonstrated that the repeated inhalation of allergen on three occasions separated by 48 hours lead to initial bronchoconstriction and an eosinophilic inflammatory response which was detectable four days after the last challenge. Similar repeated inhalation challenges with methacholine, which induced a similar degree of initial bronchoconstriction, did not lead to eosinophilic inflammation. Inhalation challenge with saline or salbutamol followed by methacholine did not induce either bronchoconstriction or inflammation.

As the bronchoconstriction and eosinophilic inflammatory responses are correlated in the allergen group and have been dissociated in the methacholine group, it is now possible to investigate their relative roles in inducing airway remodelling in our asthmatic subjects.

5.1 Background

As described above, previous published work has demonstrated that single inhaled allergen challenge in human volunteers results in cellular inflammation and remodelling changes (Kariyawasam 2007). Repeated bronchoconstriction may itself induce airway changes including increased airway permeability and possibly remodelling by a mechanism of reduction of intercellular volume in the constricted epithelium (Halldorsdottir 1997, Tschumperlin 2004) though this has not been examined *in vivo*. Although it is well recognised that mechanical stress can induce changes in cultured bronchial epithelial cells (Tschumperlin 2003) co-culture models (Choe 2006) and animal systems (Tschumperlin 2004) it is not known whether the effect of repeated bronchoconstriction and subsequent epithelial mechanical stress can induce epithelial activation or remodelling in human subjects.

5.1.1 Eosinophils and mechanical stress as potential initiators of airway remodelling

As discussed in detail in Chapter 1, eosinophils and airway bronchoconstriction are both implicated in the process of airway remodelling. Briefly, eosinophils are virtually pathognomonic of asthma and are present from very early in the disease (Sagiani 2007). Following administration of a monoclonal antibody to IL5, mepolizumab, the numbers of circulating eosinophils and also the number of eosinophils within the airway are decreased (Flood-Page 2003, 2007). Mepolizumab also reduces asthma exacerbation frequency and improves asthma related quality of life scores in asthmatics (Haldar 2009, Nair 2009). Following administration of anti-IL5 antibody for 3 months in human atopic asthmatic volunteers, there was a reduction in the basement membrane deposition of a variety of structural proteins including tenascin, lumican, and procollagen III. Tenascin levels were reduced to levels seen in normal individuals (Flood-Page 2003). Such results strongly implicate the eosinophil in the mechanisms underlying airway remodelling.

Although not yet investigated *in vivo*, the role of mechanical stress on the epithelium of the respiratory tract during bronchoconstriction has been investigated by a variety of models *in vitro* (Ressler 2000, Swartz 2001, Tschumperlin 2003, Park 2009). These experiments have shown that the mechanical stress associated with bronchoconstriction is sufficient to induce the production of a variety of profibrotic cytokines from the cultured airway epithelium, which can then act on cultured fibroblasts to induce collagen production (Tschumperlin 2003). In addition, repeated physical stress designed to mimic repeated daily bronchoconstriction has caused epithelial metaplasia *in vitro* (Park 2009).

With the current group of volunteers, it is now possible to investigate the relative roles of bronchoconstriction in the presence and absence of eosinophilic

inflammation on epithelial activation and also airway remodelling as measured using collagen deposition, airway cell turnover and epithelial metaplasia.

5.1.2 Epithelial activation in asthma

Asthma may be considered as a chronic wound healing disease, where the epithelial-mesenchymal trophic unit (EMTU) of the airway wall is chronically activated. This chronic activation leads to incomplete and ongoing repair to the airway with continued activation of many components of the airway wall, including the epithelium (reviewed in Holgate 2000 and 2007). This asthma paradigm requires that the epithelium be activated repeatedly by a variety of stimuli, and this has been demonstrated with oxidant stress (Bucchieri 2002), respiratory virus infection (Bayram 2002) and allergen challenge (Flood-Page 2003). Whether or not bronchoconstriction alone may activate the epithelium *in vivo* has yet to be established.

The activation of the epithelium in asthma may be measured in several ways. One of the most extensively measured compounds is transforming growth factor beta ($TGF\beta$) which has been shown to be upregulated in asthma in humans at baseline and following allergen challenge. In addition to this marker of epithelial activation, there are also some less well studied markers including osteopontin (OPN) and one of the Adipokine family of molecules, resistin-like molecule beta ($RELM\beta$). OPN has been shown to be upregulated in mouse models of asthma, though has yet to be investigated in human allergen challenge (Simoes 2009); similarly $RELM\beta$, a molecule originally identified in mouse models, has been shown to be necessary and sufficient for remodelling in mice (Stutz 2003). The turnover of cells in the epithelium of asthmatics has also been shown to be abnormal, and changes significantly with allergen challenge (Ricciardolo 2003); this cell turnover may be assessed using a marker of proliferation such as ki-67. All of these markers of epithelial activation will therefore be examined in the four groups of volunteers in this study.

5.1.3 Ki-67

Ki-67 is a monoclonal antibody raised against an originally unknown protein which was found to be associated with cellular proliferation (Gerdes 1983). The protein itself is now generally known as Ki-67 protein, and it is known to be exclusively expressed in proliferating cells and is tightly regulated, most probably by rapid proteolytic pathways. It has also been shown that Ki-67 protein is not simply associated with cellular proliferation but, using antisense oligonucleotides to Ki-67 protein RNA, is required for cell turnover, although its exact function remains unknown (Schulter 1993, Endl 2000). Ki-67 antibody is most commonly used clinically in the assessment of prognosis in human cancers (Woo 2009), though it has also been investigated in airways disease. Ki-67 positive cells are increased in the respiratory epithelium of patients with severe COPD, though there is no increase in smokers. In severe COPD the number of Ki-67 cells in the epithelium is correlated with the FEV₁:FVC ratio (Pilette 2007). Examination of the epithelium of adult asthmatics using a different marker of cellular proliferation (proliferating cell nuclear antigen) has shown that there is no excess proliferation in the asthmatic patients compared to normals, though again this study showed that there was an increase in the cellular turnover in patients with chronic bronchitis (Demoly 1994). Following inhalation allergen challenge it has been shown that Ki-67 positive cells in the epithelium increase and are correlated with the numbers of eosinophils in the tissues (Ricciardolo 2003). The authors concluded that the allergen challenge leads to an increase in epithelial proliferation associated with the eosinophilic inflammation following allergen challenge. It has not been investigated whether bronchoconstriction alone may induce such a proliferative change.

5.1.4 Measurement of airway remodelling

Although epithelial activation may occur in the paradigm of the epithelial-mesenchymal unit, this activation is of interest relating to the downstream effects on other components of the airway wall, specifically on the remodelling

of the airway. Airway remodelling refers to alterations in the structure of the airway, and includes thickening of the basement membrane, subepithelial fibrosis, goblet cell and submucosal gland enlargement, increased smooth muscle mass and increased vascularity. These alterations may be measured in various ways (reviewed in Bergeron 2007), but here the primary method of measurement is immunohistochemistry for a selection of these changes, specifically basement membrane and subepithelial fibrosis by examination of basement membrane thickness and percentage of submucosal area staining positive for collagen III. In addition periodic acid Schiff reagent will be used to examine for possible hypertrophy of mucus glands. Although no direct measure of neovascluarisation or smooth muscle expansion will be made, a measure of submucosal cell turnover will be made using Ki-67 antibody as above.

5.2 Aims

To investigate the hypothesis that repeated inhalation challenge with allergen and methacholine, matched for initial bronchoconstrictive response, will induce different remodelling responses in the airway.

Additionally to assess the expression of RELM β in the airways of volunteers with different degrees of airway disease.

5.3 Methods and Results

5.3.1 Volunteers

Human volunteers were recruited, characterised and challenged as described in Chapter 2.

5.3.2 Samples

Serum, bronchoalveolar lavage fluid and bronchial biopsies were taken prior to and four days after repeated inhalation challenge as previously described. Bronchial biopsies were examined by immunohistochemistry for a variety of markers of remodelling and also following RNA extraction, for mRNA expression. BAL fluid and serum were examined by ELISA.

5.3.3 Immunohistochemistry

Immunohistochemistry was performed as previously described using antibodies detailed in Chapter 2.

Epithelial activation markers

Epithelial activation markers measured were TGF β and RELM β (expressed as percentage staining of the epithelium) and the number of Ki-67 positive proliferative cells per mm length of epithelium.

TGF β

Typical staining using an antibody to panTGF (all isoforms) is shown in Figure 5-1. Prior to inhalation challenge there was no significant difference between the four challenge groups with the median (IQR) percentage staining in the allergen group of 0.98 (0.90 - 3.25)%, in the methacholine group of 2.00 (1.13 - 3.26), in the saline group 1.74 (0.98 - 2.48) and in the salbutamol / methacholine group 1.80 (0.67 - 2.32) ($p=0.76$). Following inhalation challenge there was a significant increase in the percentage staining in both the allergen and methacholine groups, with the median (IQR) percentage staining increasing to 3.18 (0.90 - 4.78)% in the allergen group ($p=0.01$) and in the methacholine group to 4.03 (3.28 - 6.75) ($p=0.003$) whilst there was no significant change in the saline group (post challenge 2.29 (0.39 - 3.28) % ($p=0.64$)) or in the salbutamol / methacholine group (post challenge 1.54 (0.67 - 2.54) % ($p=0.79$)).

as shown in. When the difference between the post and pre challenge staining was calculated and compared, there was a significant difference between the four groups ($p=0.01$). Between group comparisons using two groups and the Mann-Whitney Test show that there was no difference between the change in staining in the allergen and methacholine groups ($p=0.14$), and also not between the allergen and the saline challenges ($p=0.34$), though there was a difference between the allergen and the salbutamol / methacholine groups ($p=0.02$). The methacholine challenge was significantly different to both saline and salbutamol / methacholine challenges ($p=0.03$ and $p=0.02$ respectively), whilst there was no difference between the saline and salbutamol / methacholine challenges ($p=0.76$). These results are shown in Table 5-1 and in Figure 5-2.

Figure 5-1 Immunohistochemical staining of TGF β using a anti pan TGF β antibody (arrow). Chromogen DAB. Bar = 100 μ m.

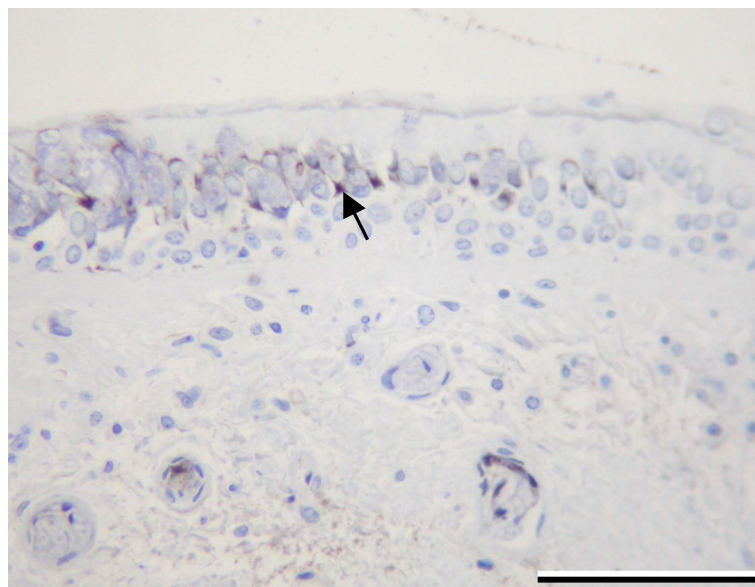


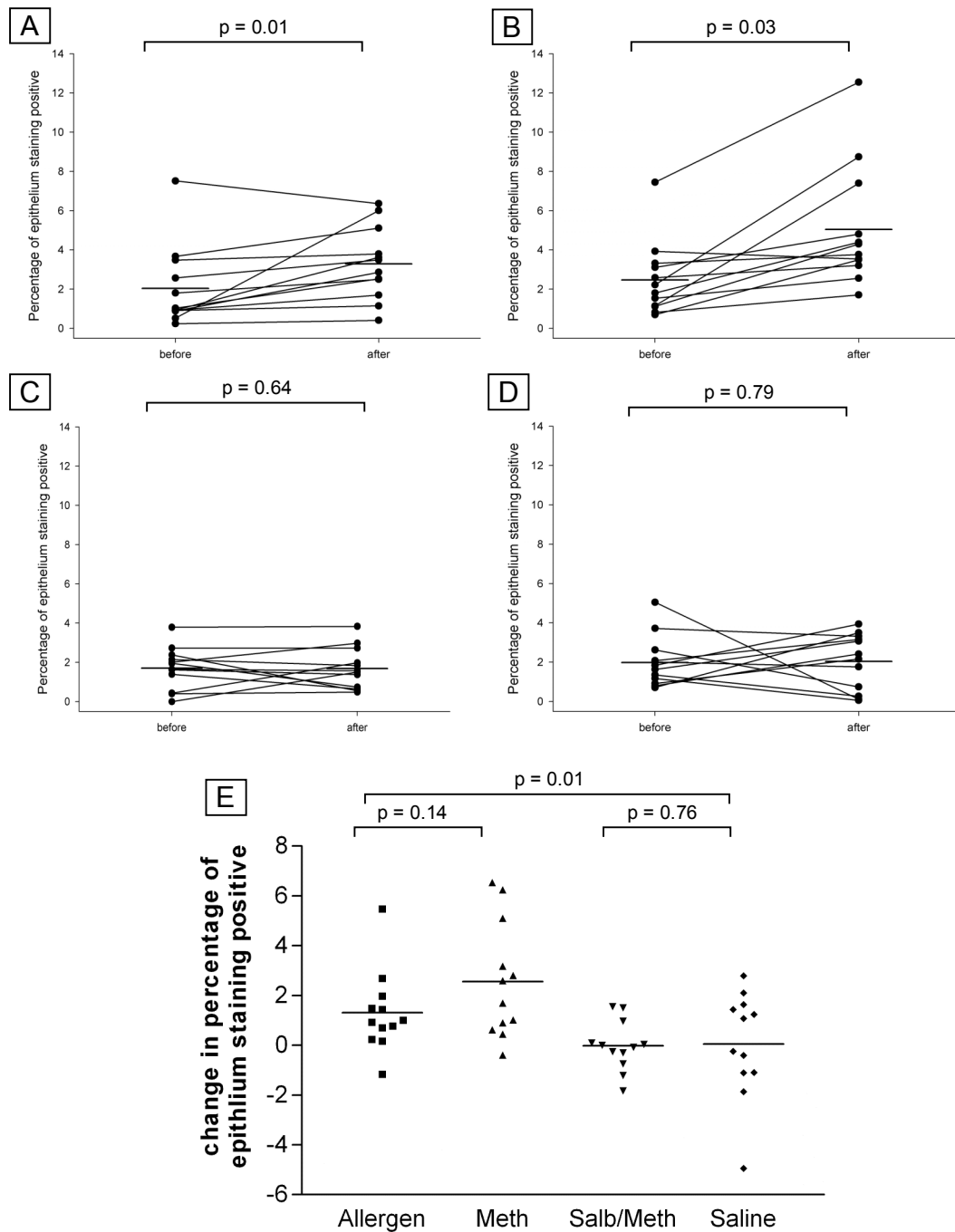
Table 5-1 Epithelial immunoexpression of TGF β expressed as percentage of total epithelial area before and after repeated inhaled challenges.

TGF β percentage staining of epithelium

	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	0.98 (0.90 - 3.25)	3.18 (0.90 - 4.78)	0.012	0.85 (0.25 - 1.85)
Methacholine	2.00 (1.13 - 3.26)	4.03 (3.28 - 6.75)	0.003	2.15 (0.69 - 4.62)
Saline	1.74 (0.98 - 2.48)	2.29 (0.39 - 3.28)	0.638	0.42 (-1.10 - 1.58)
Salbutamol / methacholine	1.80 (0.67 - 2.32)	1.54 (0.67 - 2.54)	0.790	0.04 (-0.63 - 0.75)
p value	0.757	0.01		0.011

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. Comparison of two groups by Mann-Whitney Test; allergen v methacholine p=0.14, allergen v saline p=0.34, allergen v salbutamol p=0.02, methacholine v saline p=0.03, methacholine v salbutamol p=0.02, salbutamol v saline p=0.76.

Figure 5-2 Immunoexpression of transforming growth factor beta (TGF β) expressed as percentage of total epithelial area following repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine (meth), C - salbutamol followed by methacholine (salb/meth) and D - saline. Change in percentage staining of TGF β is shown in panel E. Bars show mean.



RELM β

No previous immunohistochemistry work had been performed using antibodies to human RELM β , therefore several antibodies were tried, and optimal concentrations and conditions for staining ascertained as described above. RELM β was principally identified in the epithelium, with some positive staining in the vascular endothelium as is shown at Figure 5-3. Isotype controls were performed, and demonstrated no staining, in addition, preabsorption of the antibody with recombinant RELM β protein also showed no staining as described and shown in Chapter 2. These findings concord with results from mouse models where RELM β is expressed in lung epithelial cells and inflammatory cells (Homer 2007). Prior to inhalation challenges there was similar staining for RELM β in the epithelium of the biopsies from the four challenge groups, median (IQR) percentage staining was 2.15 (0.77 - 3.19)% in the allergen group, 4.21 (1.30 - 5.09) in the methacholine group, 3.65 (2.07 - 6.10) in the saline group and 3.88 (2.38 - 4.41) in the salbutamol / methacholine group ($p=0.32$). Following inhalation challenges there was a significant increase in the percentage of epithelium staining positive for RELM β in both the allergen and methacholine groups with the median (IQR) in the allergen group following challenge increasing to 4.35 (3.02 - 6.07) % ($p=0.01$) and in the methacholine group to 6.16 (4.72 - 8.77) ($p=0.01$), whilst there was no increase in the saline or salbutamol / methacholine groups as shown in Table 5-2. When comparing the difference between the pre and post inhalation challenge results between groups there was no significant difference. These results are also shown in Figure 5-4.

Figure 5-3 Immunohistochemical staining of resistin like molecule beta (RELM β) using an anti RELM β antibody (arrow). Chromogen DAB. Bar = 100 μ m.

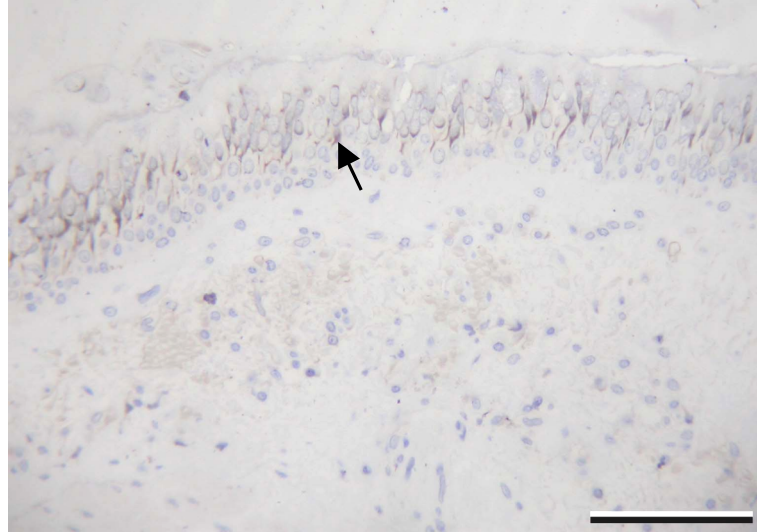


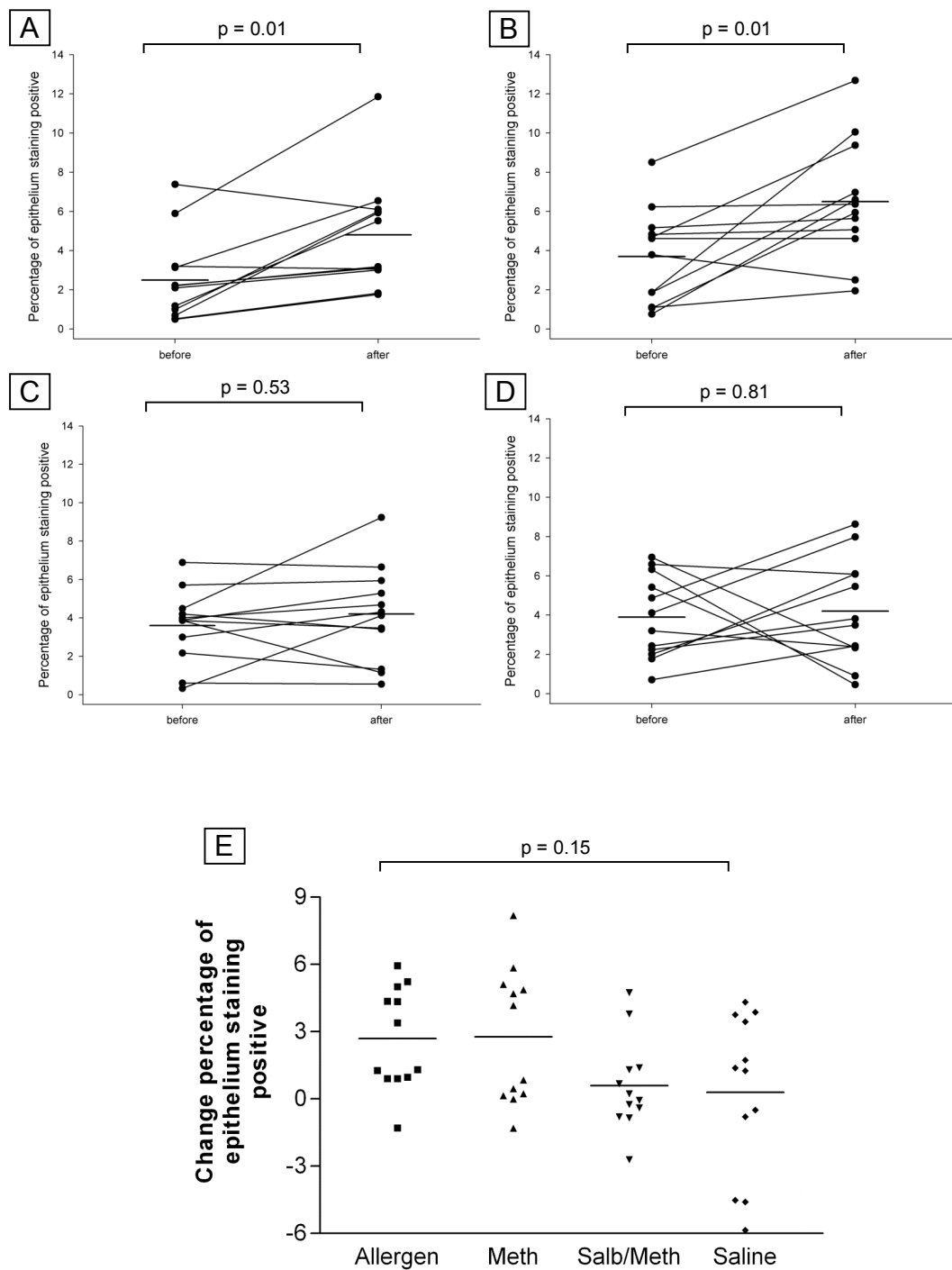
Table 5-2 Epithelial immunoexpression of RELM β expressed as percentage of total epithelial area before and after repeated inhaled challenges.

RELM β percentage staining of epithelium

	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	2.15 (0.77 - 3.19)	4.35 (3.02 - 6.07)	0.01	1.29 (0.91 - 4.84)
Methacholine	4.21 (1.30 - 5.09)	6.16 (4.72 - 8.77)	0.01	2.51 (0.16 - 5.04)
Saline	3.65 (2.07 - 6.10)	3.65 (2.35 - 6.10)	0.53	1.31 (-3.59 - 3.68)
Salbutamol / methacholine	3.88 (2.38 - 4.41)	4.21 (1.84 - 5.77)	0.81	0.08 (-0.69 - 1.37)
p value	0.32	0.18		0.15

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Figure 5-4 Immunoexpression of resistin like molecule beta (RELM β) expressed as percentage of total epithelial area following repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine (meth), C - salbutamol followed by methacholine (salb/meth) and D - saline. Change in percentage staining of RELM β is shown in panel E. Bars show mean.



Epithelial Ki-67

The cellular proliferation marker Ki-67 was stained for in bronchial biopsies as described above, typical staining is shown in Figure 5-4. Prior to inhalation challenge there were similar numbers of cells staining positive for Ki-67 per mm length of epithelium. Median (IQR) numbers of cells per mm in the allergen group was 1.97 (0.25 - 4.85) cells/mm, in the methacholine group 0.83 (0.00 - 3.21), in the saline group 2.32 (0.34 - 5.91) and in the salbutamol / methacholine group 3.76 (2.55 - 8.38) ($p=0.09$). Following the inhalation challenges there was a significant increase in the number of positive cells per mm of epithelium in the allergen and the methacholine groups. In the allergen group the median (IQR) number of positive cells per mm increased to 23.4 (4.69 - 29.64) cells/mm ($p=0.01$) and in the methacholine group increased to 7.9 (4.50 - 16.30) ($p=0.01$) whilst there was no significant change in the saline group (1.90 (0.00 - 3.72) ($p=0.58$)) or in the salbutamol / methacholine group (3.02 (2.00 - 9.32) ($p=0.70$)). When the difference between the post and pre challenge cell counts per mm of epithelium were calculated there was a significant difference between the four groups ($p=0.001$) with the allergen group having a median (IQR) difference of 17.53 (2.31 - 24.73), the methacholine group 6.27 (2.01 - 12.31), the saline group 0.00 (-4.59 - 1.70), and the salbutamol group -0.18 (-2.18 - 1.78). Comparing between pairs of groups using the Mann-Whitney Test showed there was no significant difference in the change in ki-67 staining between the allergen and methacholine groups ($p=0.16$) whilst both the allergen and methacholine groups were significantly different to both the saline and the salbutamol / methacholine groups (as shown in Table 5-3). There was no difference between the saline and salbutamol / methacholine groups ($p=0.84$). These data are also shown in Figure 5-6.

Figure 5-5 Immunohistochemical staining of cells with anti Ki-67 antibody (arrow). Chromogen DAB. Bar = 100 μ m.

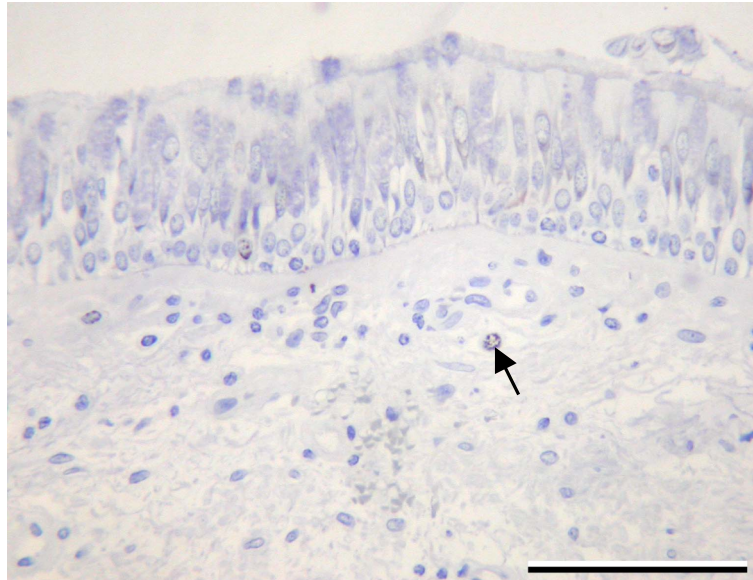
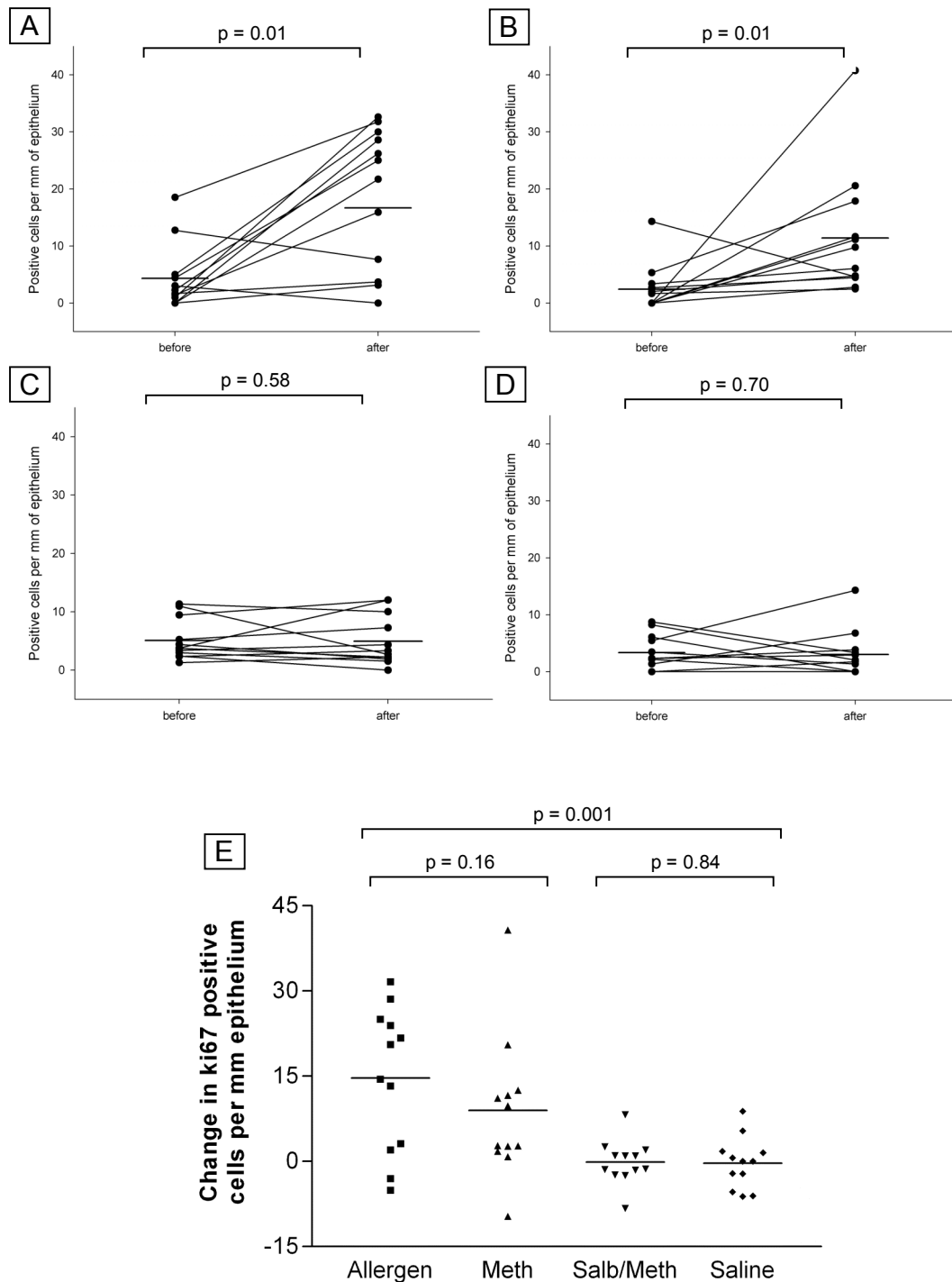


Table 5-3 Ki-67 positive cells per mm length of epithelium before and after repeated inhaled challenges.

	Ki 67 staining (cells per mm length of epithelium)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	1.97 (0.25 - 4.85)	23.4 (4.69 - 29.64)	0.010	17.53 (2.31 - 24.73)
Methacholine	0.83 (0.00 - 3.21)	7.9 (4.50 - 16.30)	0.010	6.27 (2.01 - 12.31)
Saline	2.32 (0.34 - 5.91)	1.90 (0.00 - 3.72)	0.575	0.00 (-4.59 - 1.70)
Salbutamol / methacholine	3.76 (2.55 - 8.38)	3.02 (2.00 - 9.32)	0.695	-0.18 (-2.18 - 1.78)
p value	0.091	0.001		0.001

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. Comparison of two groups by Mann-Whitney Test; allergen v methacholine p=0.16, allergen v saline p=0.03, allergen v salbutamol p =0.007, methacholine v saline p=0.05, methacholine v salbutamol p=0.05, salbutamol v saline p=0.84.

Figure 5-6 Ki-67 positive cells per millimetre of bronchial epithelium following repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine (meth), C - salbutamol followed by methacholine (salb/meth) and D - saline. Change number of Ki-67 positive cells is shown in panel E. Bars show mean.



Epithelial MMP9 cells

Typical staining for cells positive to MMP9 is shown in Figure 5-7. There were very few MMP9 positive cells in the epithelium of the biopsies either before or after inhalation challenge. There were no significant difference between or within any groups at any point as shown in Table 5-4.

Figure 5-7 Immunohistochemical staining of cells with anti MMP9 antibody (arrow). Chromogen DAB. Bar = 100 μ m.

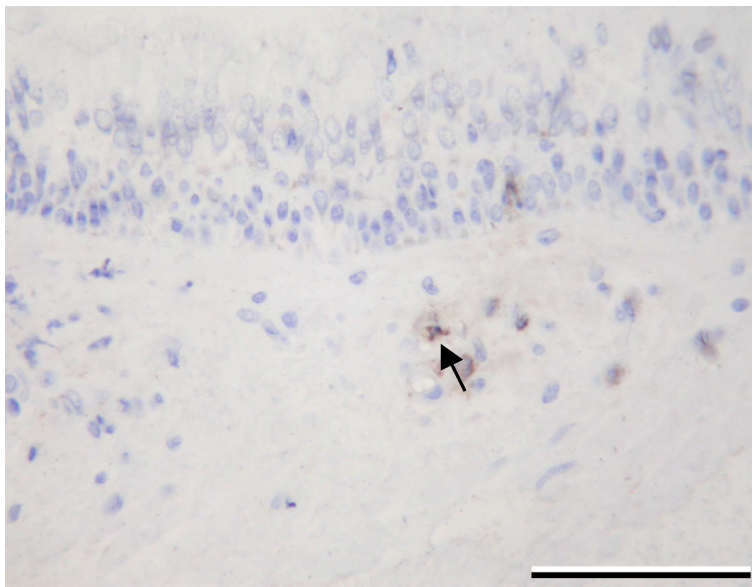


Table 5-4 MMP9 positive cells per mm length of epithelium before and after repeated inhaled challenges.

	MMP9 positive cells per mm epithelium			
	Pre challenge	Post challenge	p value within group	Change (Δ)
Allergen	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	1.00	0.0 (0.0 - 0.0)
Methacholine	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.66	0.0 (0.0 - 0.0)
Saline	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.31	0.0 (0.0 - 0.0)
Salbutamol / methacholine	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.32	0.0 (0.0 - 0.0)
p value	0.51	0.54		0.58

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

5.3.4 Remodelling markers

The markers of remodelling measured were; Basement membrane thickness (μm), percentage of submucosa stained with collagen III antibody, the numbers of proliferating cells per mm^2 of submucosa and the percentage of the epithelium staining positive for mucins. The numbers of submucosal MMP9 positive cells per mm^2 was also calculated.

Basement membrane thickness

Using biopsies stained with an antibody against collagen III (Figure 5-8) the basement membrane thickness was measured prior to inhalation challenge in the four challenge groups as previously described. Prior to inhalation challenge there was no significant difference between the four groups with median (IQR) basement membrane thickness in the allergen group 7.47 (7.01 - 8.81) μm , in the methacholine group 7.96 (6.59 - 9.28), in the saline group 7.48 (6.12 - 9.29) and in the methacholine group 10.76 (7.78 - 13.51) ($p=0.14$). Following inhalation challenge there was a significant thickening of the membrane in the allergen and methacholine groups, with allergen increasing to a median (IQR) of 10.54 (7.95 - 11.58) μm ($p=0.01$) and the methacholine group increasing to 9.73 (8.13 - 12.03) ($p=0.005$) whilst there was no significant change in the either the saline groups (7.20 (5.66 - 8.57) $p=0.09$) or the salbutamol / methacholine group (10.07 (7.84 - 12.76) $p=0.94$). Comparing the change in basement membrane thickness, there was a significant difference between the four groups ($p<0.001$) with group pair comparisons using Mann-Whitney Testing showing that there was no significant difference between the allergen and methacholine groups in terms of change in basement membrane thickness ($p=0.76$), whilst each of the allergen and methacholine groups was significantly different to each of the saline and salbutamol / methacholine challenges. The saline and salbutamol / methacholine challenges were not different to each other ($p=0.18$) as shown at Table 5-5 and Figure 5-9.

**Figure 5-8 Immunohistochemical staining of collagen III (arrow).
Chromogen DAB. Bar = 100 μ m.**

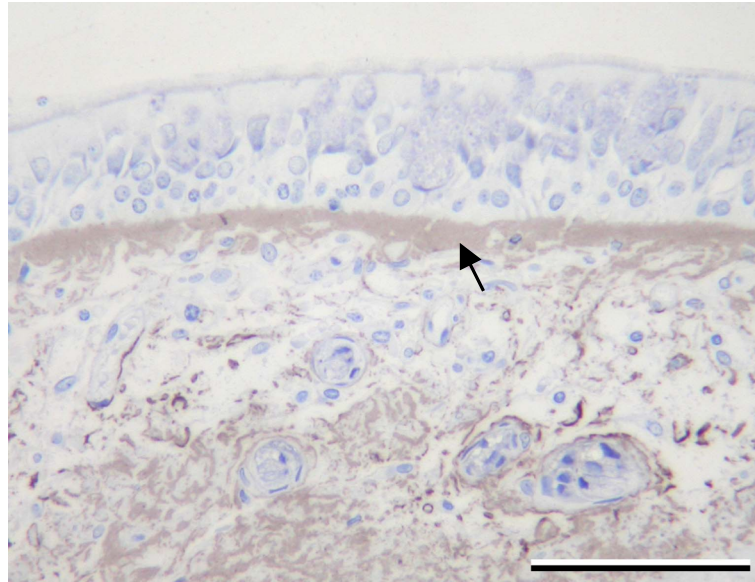
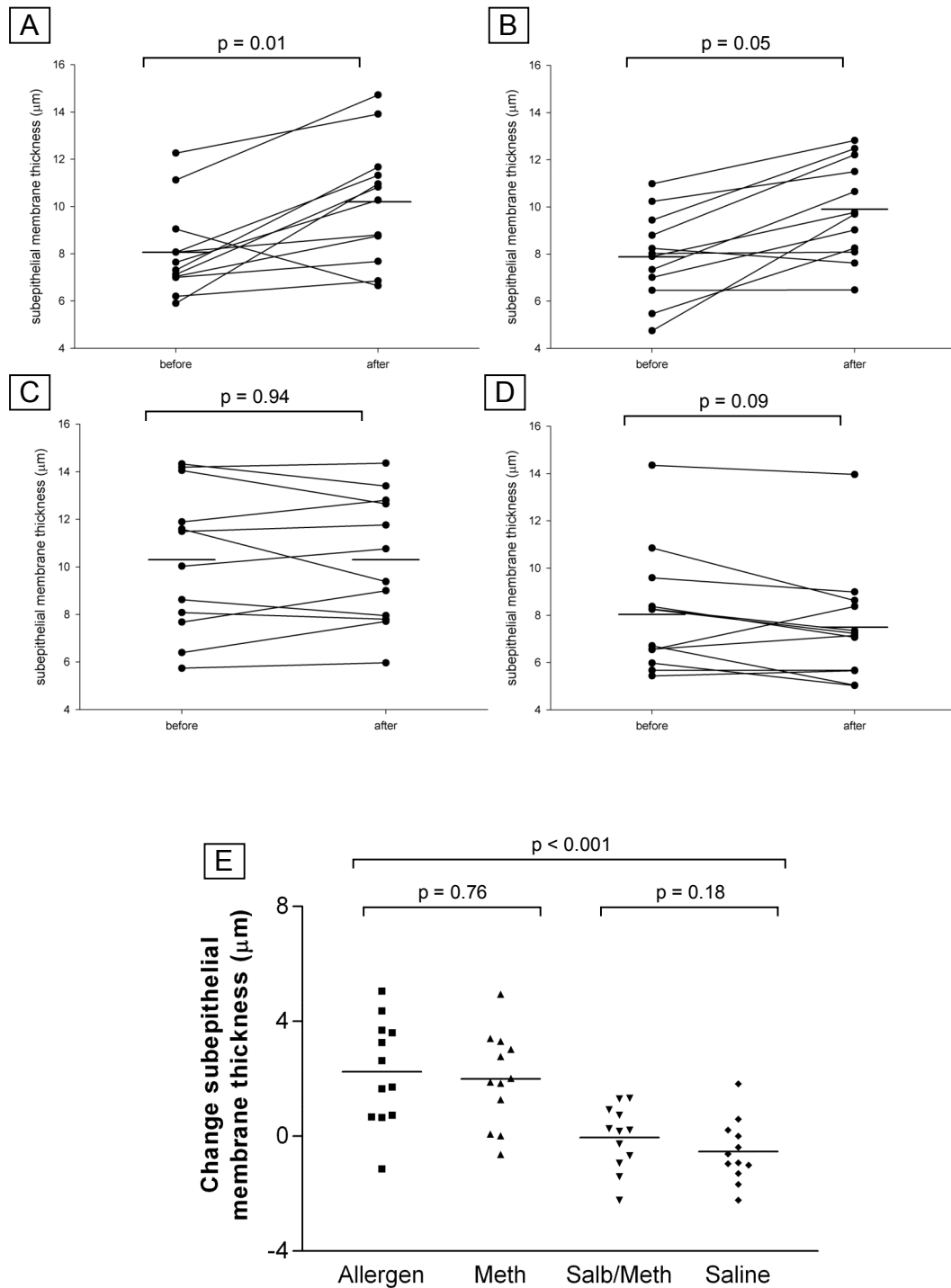


Table 5-5 Subepithelial basement membrane thickness in bronchial biopsies before and after repeated inhaled challenges.

	subepithelial collagen thickness (μm)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	7.47 (7.01 - 8.81)	10.54 (7.95 - 11.58)	0.010	2.17 (0.70 - 3.67)
Methacholine	7.96 (6.59 - 9.28)	9.73 (8.13 - 12.03)	0.005	1.94 (0.37 - 3.24)
Saline	7.48 (6.12 - 9.29)	7.20 (5.66 - 8.57)	0.091	-0.77 (-1.23 - 0.17)
Salbutamol / methacholine	10.76 (7.78 - 13.51)	10.07 (7.84 - 12.76)	0.937	0.20 (-0.86 - 0.87)
p value	0.144	0.02		<0.001

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. Comparison of two groups by Mann-Whitney Test; allergen v methacholine p=0.76, allergen v saline p=0.001, allergen v salbutamol p =0.005, methacholine v saline p<0.001, methacholine v salbutamol p=0.005, salbutamol v saline p=0.18.

Figure 5-9 Subepithelial basement membrane thickness in bronchial biopsies (μm) before and after repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine (meth), C - salbutamol followed by methacholine (salb/meth) and D - saline. Change in thickness of collagen is shown in panel E. Bars show mean.



Periodic acid Schiff staining

Mucins in the epithelium were stained using periodic acid-Schiff (PAS) reagent as shown in Figure 5-10, the percentage of the epithelium positive for PAS was calculated for each of the four groups prior to and following inhalation challenge. Prior to challenge there was no significant difference between the four groups with the allergen challenge group having a median (IQR) percentage staining of 2.60 (0.33 - 5.10)%, the methacholine group of 1.75 (0.97 - 2.96), the saline group of 5.08 (2.24 - 7.67) and the salbutamol / methacholine group of 3.72 (0.66 - 5.44) ($p=0.15$). Following inhalation challenge there was an increase in the allergen and methacholine groups, with the median (IQR) increasing in the allergen group to 5.47 (2.35 - 8.22) (though this was not significant within the group ($p=0.10$)) and in the methacholine group to 5.00 (3.12 - 9.37) ($p=0.03$). In the saline group there was a significant decrease in the mucin staining to 2.50 (1.78 - 6.25)% ($p=0.03$) whilst the salbutamol / saline group was unchanged at 1.50 (0.89 - 3.45) % ($p=0.48$). When comparing the difference between the post and pre challenge percentages the median (IQR) differences were 2.17 (1.03 - 4.77) in the allergen group, 2.13 (1.14 - 7.96) in the methacholine group, -1.82 (-4.48 - 0.04) in the saline group and -0.18 (-3.08 - 0.97) in the salbutamol group. These changes were significantly different across the four groups ($p=0.003$). When comparing pairs of groups by the Mann-Whitney Test, the allergen and methacholine groups change in percentage staining was not significantly different ($p=0.59$) whilst each of the allergen and methacholine groups were significantly different to each of the saline and salbutamol / methacholine groups. There was no significant difference between the saline and salbutamol / methacholine groups for this parameter ($p=0.35$). These results are shown at Table 5-6 and Figure 5-11.

Figure 5-10 Histochemical staining of mucins using periodic acid-Schiff reagent (arrow). Bar = 100 μ m.

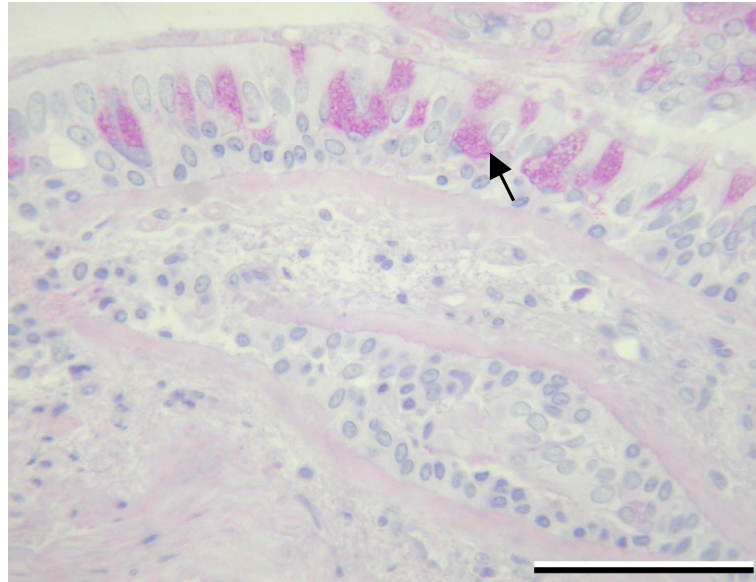
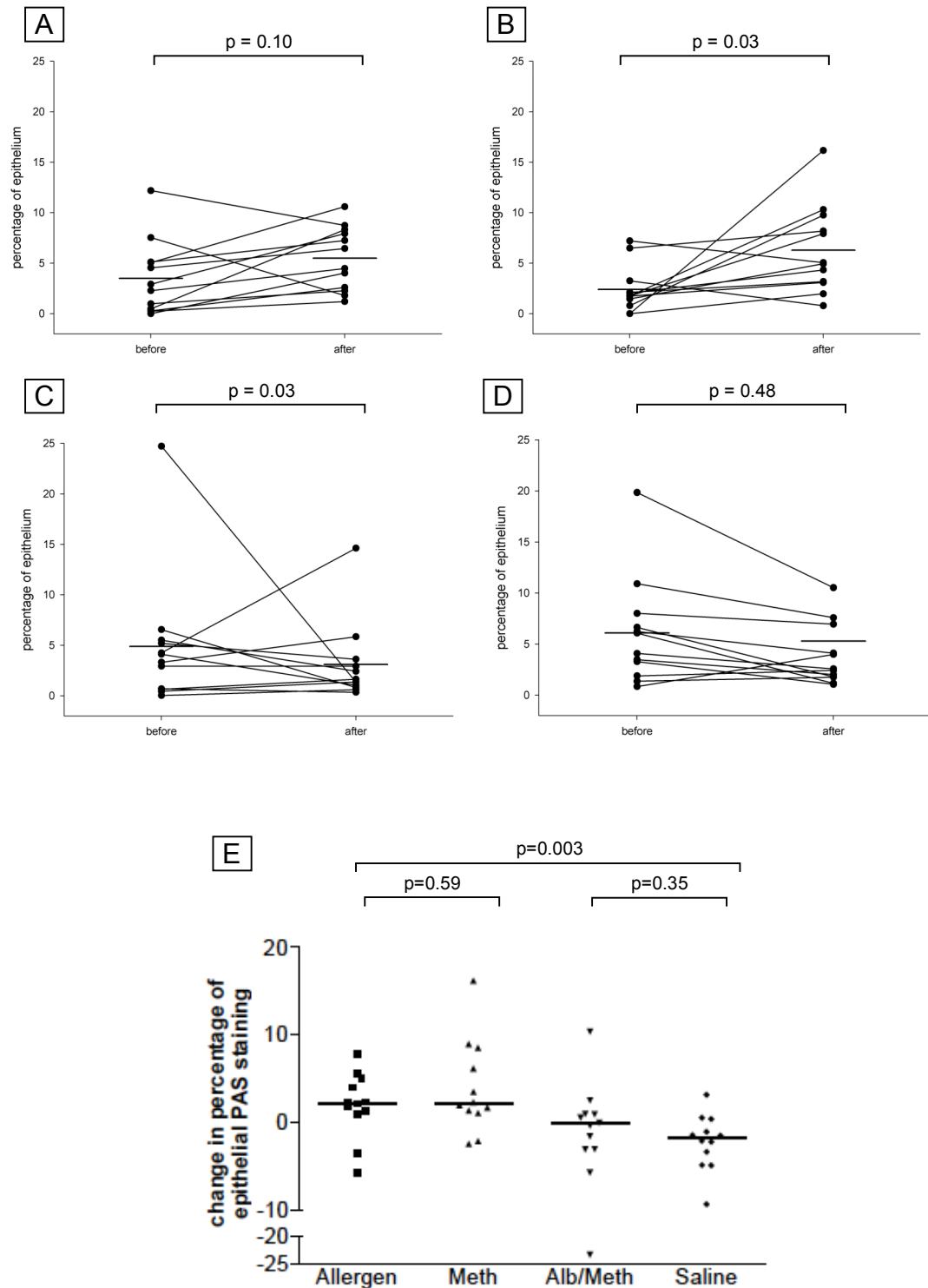


Table 5-6 Positive periodic acid - Schiff (PAS) staining in bronchial biopsies as percentage of total epithelial area before and after repeated inhaled challenge.

	PAS staining as percentage of total epithelial area			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	2.60 (0.33 - 5.10)	5.47 (2.35 - 8.22)	0.10	2.17 (1.03 - 4.77)
Methacholine	1.75 (0.97 - 2.96)	5.00 (3.12 - 9.37)	0.03	2.13 (1.14 - 7.96)
Saline	5.08 (2.24 - 7.67)	2.50 (1.78 - 6.25)	0.03	-1.82 (-4.48 - 0.04)
Salbutamol / methacholine	3.72 (0.66 - 5.44)	1.50 (0.89 - 3.45)	0.48	-0.18 (-3.08 - 0.97)
p value	0.15	0.026		0.003

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. Comparison of two groups by Mann-Whitney Test; allergen v methacholine p=0.59, allergen v saline p=0.007, allergen v salbutamol p =0.045, methacholine v saline p=0.02, methacholine v salbutamol p=0.014, salbutamol v saline p=0.35.

Figure 5-11 Percentage of epithelium staining positive with periodic acid - Schiff (PAS) reagent following repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine (meth), C - salbutamol followed by methacholine (salb/meth) and D - saline. Change in percentage positive PAS staining is shown in panel E. Bars show mean.



Submucosal Ki-67 cells

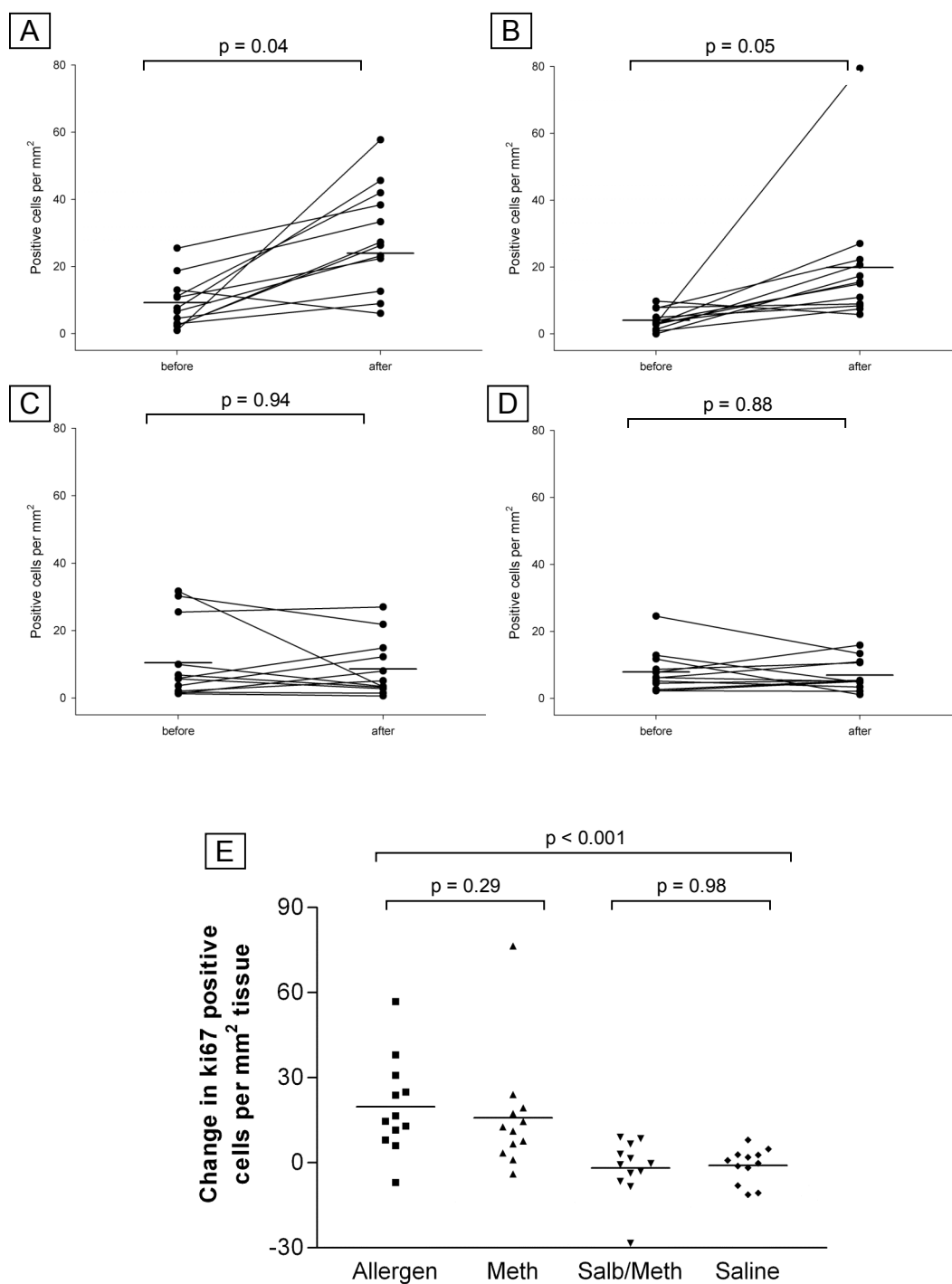
The numbers of Ki-67 positive cells per mm² of submucosa tissue were counted in the biopsies before and after the inhalation challenges; prior to the inhalation challenges there were no differences between the four groups. In the allergen challenge group the median (IQR) pre challenge Ki-67 cells per mm² of submucosal tissue was 7.10 (2.58 - 12.53) cells/ mm², in the methacholine group 3.18 (1.71 - 7.01), in the saline group 6.19 (3.12 - 11.00) and in the salbutamol / methacholine group 5.80 (1.81 - 21.61) (p=0.32). Following inhalation challenge there was a significant increase in both the allergen (26.78 (15.06 - 41.05) cells/ mm² (p=0.04) and the methacholine groups (15.25 (8.57 - 21.83) cells/ mm² (p=0.05), whilst there was no significant increase in the saline or salbutamol / methacholine groups (Table 5-7). The change in the numbers of positive cells between the post and pre challenge biopsies was significantly different across the four groups (p<0.001) with the median (IQR) change in the allergen group 15.58 (8.94 - 29.36), in the methacholine group 11.88 (4.27 - 18.81), and in the saline group 0.35 (-6.50 - 2.80) and in the salbutamol / methacholine group -0.48 (-5.85 - 5.76). Comparison between pairs of groups using the Mann-Whitney Test showed no difference between the allergen and methacholine groups (p=0.29) or between the saline and salbutamol / methacholine groups (p=0.98). There was however a significant difference between both the allergen and methacholine groups and both the saline and salbutamol/methacholine groups as shown in Table 5-7 and Figure 5-12.

Table 5-7 Number of Ki-67 positive cells per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges.

	Ki 67 positive cells per mm ² of submucosal tissue			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	7.10 (2.58 - 12.53)	26.78 (15.06 - 41.05)	0.04	15.58 (8.94 - 29.36)
Methacholine	3.18 (1.71 - 7.01)	15.25 (8.57 - 21.83)	0.05	11.88 (4.27 - 18.81)
Saline	6.19 (3.12 - 11.00)	5.22 (3.75 - 10.87)	0.94	0.35 (-6.50 - 2.80)
Salbutamol / methacholine	5.80 (1.81 - 21.61)	4.25 (2.90 - 14.23)	0.88	-0.48 (-5.85 - 5.76)
p value	0.32	<0.001		<0.001

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. Comparison of two groups by Mann-Whitney Test; allergen v methacholine p=0.29, allergen v saline p<0.001, allergen v salbutamol p<0.001, methacholine v saline p=0.001, methacholine v salbutamol p=0.003, salbutamol v saline p=0.98.

Figure 5-12 Ki-67 positive cells per square millimetre of bronchial submucosa before and after repeated inhaled challenges. Panel A - Allergen challenges, B - methacholine (meth), C - salbutamol followed by methacholine (salb/meth) and D - saline. Change number of Ki-67 positive cells is shown in panel E. Bars show mean.



MMP9 cells

The number of MMP9 positive cells were counted per mm² of submucosal tissue before and after inhalation challenge; There were no prechallenge differences between the four groups; median (IQR) cell numbers were 5.44 (1.49 - 10.20) cells/ mm² in the allergen group, 7.20 (2.64 - 19.45) in the methacholine group, 4.41 (2.01 - 14.08) in the saline group and 2.53 (0.21 - 13.39) in the salbutamol/ methacholine group (p=0.48). Following the inhalation challenges there was a significant decrease in the numbers of MMP9 positive cells per mm² of submucosa in the allergen group to 2.75 (0.20 - 5.66) (p=0.03) however there was no significant difference when comparing the between group change in numbers of cells as shown in Table 5-8.

Table 5-8 Number of MMP9 positive cells per square mm of submucosal tissue in bronchial biopsies before and after repeated inhaled challenges.

MMP9 positive cells per mm ² of submucosal tissue				
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	5.44 (1.49 - 10.20)	2.75 (0.20 - 5.66)	0.03	-2.40 (-5.73 - 0.00)
Methacholine	7.20 (2.64 - 19.45)	6.28 (1.10 - 14.19)	0.79	-0.64 (-6.70 - 3.93)
Saline	4.41 (2.01 - 14.08)	5.72 (3.23 - 12.08)	0.64	0.67 (-0.46 - 5.45)
Salbutamol / methacholine	2.53 (0.21 - 13.39)	5.38 (1.33 - 9.56)	0.51	0.53 (-3.09 - 5.33)
p value	0.48	0.50		0.23

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. MMP - matrix metalloproteinase.

Submucosal collagen percentage

The percentage of the submucosal area of the biopsies staining positive for Collagen III was calculated as previously described. There were no differences between the percentage of submucosal collagen staining before or after inhalation challenge within or between any of the four groups at any point as shown in Table 2-1.

Table 5-9 Percentage of submucosal tissue staining positive for collagen III before and after repeated inhaled challenges.

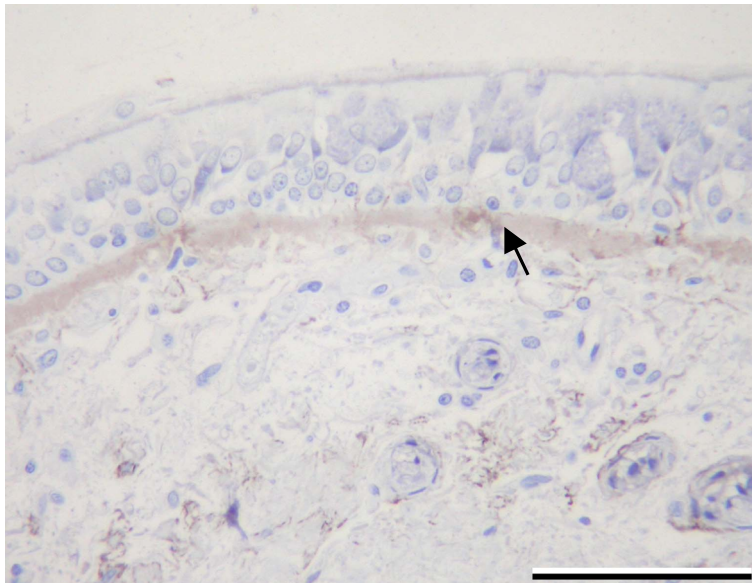
	Collagen submucosal percentage staining			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	15.68 (12.26 - 20.23)	15.92 (13.53 - 24.52)	0.12	2.06 (-2.25 - 6.40)
Methacholine	17.44 (12.07 - 22.68)	19.16 (16.73 - 22.90)	0.08	3.28 (-0.69 - 7.18)
Saline	20.07 (15.38 - 24.37)	18.74 (14.45 - 27.31)	0.51	0.42 (-4.26 - 3.59)
Salbutamol / methacholine	21.63 (19.38 - 23.65)	19.84 (18.42 - 21.48)	0.88	-1.02 (-3.27 - 1.33)
p value	0.06	0.56		0.20

n=12 for all groups. Values are median with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test.

Tenascin staining

Tenascin staining was extremely variable both between and within staining runs, and although it was examined in all biopsies taken, the results were extremely variable and were excluded. Good Tenascin staining is shown at Figure 5-13.

**Figure 5-13 Immunohistochemical staining of tenascin (arrow).
Chromogen DAB. Bar = 100 μ m.**



5.3.5 Bronchoalveolar lavage (BAL) ELISA measurements of markers of remodelling

α 2 macroglobulin, osteopontin (OPN), soluble Tumour Necrosis Factor alpha receptor 2 (sTNF α R2), Gro α , activin A and dipeptidyl peptidase 1 (cathepsin C) (DPP1) were measured in all groups before and after inhalation challenges in unconcentrated BAL fluid. In addition, surfactant protein D (SPD) was measured in the allergen, methacholine and saline groups at both time points, and Galectin 3 (Gal3) was measured in the allergen and methacholine groups. ELISA assays were performed as previously described.

α 2 macroglobulin

α 2 macroglobulin concentrations in the BAL were similar prior to inhalation challenge, median (IQR) in the allergen group was 405.8 (296.3 - 554.3) ng/ml, in the methacholine group 312.3 (189.0 - 372.3), in the saline group 432.6 (214.9 - 724.2) and in the salbutamol / methacholine group 502.4 (250.9 - 596.9) ($p=0.43$). Following inhalation challenge there was a significant increase in the concentration of α 2 macroglobulin in both the allergen and methacholine groups, with the median (IQR) increasing to 715.9 (348.6 - 2042.8) ng/ml in the allergen group ($p=0.02$) and 724.0 (416.0 - 1295.9) in the methacholine group ($p=0.05$). The saline group showed no significant difference with median (IQR) concentration following challenge of 377.9 (198.0 - 651.8) ($p=0.39$) and there was also no change in the salbutamol / methacholine group 542.4 (250.9 - 1246.3) ($p=0.14$). When the difference between pre and post challenge concentrations were calculated there was a significant difference between the four groups ($p=0.04$ for between group comparison) as shown in Table 5-10. Analysing the groups in pairs using the Mann-Whitney test demonstrated no significant difference in the change in α 2 macroglobulin concentrations in the BAL between the allergen and methacholine groups ($p=0.38$) or the saline and salbutamol / methacholine groups ($p=0.11$). There was a significant difference between both the allergen and methacholine challenge groups and the saline

group ($p=0.02$ for both pairs), but not between the allergen or the methacholine and the salbutamol / methacholine group ($p=0.38$ for allergen and 0.27 for methacholine vs salbutamol / methacholine).

Table 5-10 $\alpha 2$ macroglobulin concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of $\alpha 2$ macroglobulin (ng/ml)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	405.8 (296.3 - 554.3)	715.9 (348.6 - 2042.8)	0.023	281.7 (-7.4 - 1163.9)
Methacholine	312.3 (189.0 - 372.3)	724.0 (416.0 - 1295.9)	0.05	470.1 (35.4 - 1022.9)
Saline	432.6 (214.9 - 724.2)	377.9 (198.0 - 651.8)	0.388	-34.5 (-288.1 - 140.7)
Salbutamol / methacholine	502.4 (250.9 - 596.9)	542.4 (250.9 - 1246.3)	0.136	209.0 (-73.6 - 366.6)
p value	0.43	0.12		0.04

$n=12$ for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Comparison of two groups using Mann-Whitney Test; allergen v methacholine $p=0.93$, allergen v saline $p=0.02$, allergen v salbutamol/methacholine $p=0.38$, methacholine v saline $p=0.02$, methacholine v salbutamol/methacholine $p=0.27$, saline v salbutamol/methacholine $p=0.11$

Other BAL Elisa measurements

OPN concentrations in the BAL fluid were similar in the four groups prior to inhalation challenge with the allergen group median (IQR) concentration of 743.5 (292.5 - 1550.8) pg/ml, in the methacholine group 1054.6 (593.6 - 1354.5), in the saline group 1154.5 (1038.0 - 1460.0) and in the salbutamol / methacholine group 952.9 (676.9 - 1375.3) ($p=0.70$) as shown in Table 5-11. Following repeated inhalation challenge there was a significant fall in the OPN concentration in the salbutamol / methacholine group, falling to 952.9 (676.9 - 1375.3) pg/ml ($p=0.04$ within group difference). When the change in the concentrations between the four groups was analysed, there were no significant differences found.

sTNF α R2 concentrations were similarly measured. Prior to inhalation challenge there was no significant difference between the challenge groups, with median (IQR) sTNF α R2 concentrations of 71.7 (34.5 - 101.8) pg/ml in the allergen group, 50.6 (38.8 - 100.9) in the methacholine group, 54.1 (27.7 - 83.9) in the saline group and 53.2 (44.3 - 79.2) in the salbutamol / methacholine group ($p=0.83$). Following the inhalation challenges there was a significant fall in the concentration of sTNF α R2 in the salbutamol group to 42.5 (35.8 - 53.9) pg/ml ($p=0.04$), although comparison between groups using change in concentration showed no significant differences (Table 5-12).

GRO α , a protein similar in structure to IL-8, possesses potent neutrophil-stimulating activity. The GRO α concentrations in the BAL were similar prior to repeated inhalation challenge, with the median (IQR) in the allergen group of 2323.5 (1652.3 - 2615.8) pg/ml, in the methacholine group 2408.0 (1712.3 - 4157.0), in the saline group 1955.5 (1439.0 - 2870.8) and in the salbutamol group 2171.5 (1783.3 - 2657.5) ($p=0.90$). Following repeated challenge there was a significant increase only in the allergen group to median (IQR) of 3238.5 (2113.0 - 5751.8) pg/ml ($p=0.05$ for within group difference). Comparison of the

change in GRO α between the groups showed no significant difference (Table 5-13).

Only very low concentrations of Activin A were detectable prior to repeated inhalation challenge, and there were no significant differences between the four groups with the median (IQR) in the allergen group 0.0 (0.0 - 0.0) pg/ml, in the methacholine group 0.0 (0.0 - 278.9), in the saline group 63.0 (0.0 - 254.7) and in the salbutamol / methacholine group 200.3 (0.0 - 310.1) ($p=0.13$). Following inhalation challenges there was a significant increase in the allergen challenge group to 209.5 (0.0 - 434.2) ($p=0.03$ for within group difference), however there were no between group differences when comparing change in concentration (Table 5-14).

DPP1 when measured prior to inhalation challenge showed statistically significant differences between the four groups; the allergen group had a median (IQR) concentration of 123.3 (114.7 - 138.3) ng/ml, the methacholine group 149.1 (124.3 - 168.5), the saline group 255.4 (124.7 - 332.7) and the salbutamol / methacholine group 109.2 (88.6 - 142.4) ($p=0.02$). Following repeated inhalation challenge there were no within or between group differences when comparing absolute values, or change in concentrations (Table 5-15).

Surfactant protein D was measured in a random subset of the allergen ($n=9$), methacholine ($n=7$) and saline ($n=6$) exposure groups. There were no pre or post challenge differences between or within the groups as shown in Table 5-16. Galectin 3 was measured in a random subset of the allergen group ($n=10$) and the methacholine group ($n=4$). The Gal3 concentrations increased following both challenges (Table 5-17).

Resistin like molecule beta (RELM β) was measured in the BAL, however almost all samples were below the level of detection for the assay (35 pg/ml).

Table 5-11 Osteopontin (OPN) concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of OPN BAL (pg/ml)		p value for within group difference	Change (Δ) between challenges
	Pre challenge	Post challenge		
Allergen	743.5 (292.5 - 1550.8)	1014.7 (410.3 - 1534.5)	0.94	-1.1 (-228.5 - 215.1)
Methacholine	1054.6 (593.6 - 1354.5)	1109.5 (845.2 - 1286.5)	0.64	-113.0 (- 314.4 - 370.9)
Saline	1154.5 (1038.0 - 1460.0)	993.8 (642.2 - 1586.5)	0.43	-104.5 (- 458.6 - 272.2)
Salbutamol / methacholine	952.9 (676.9 - 1375.3)	696.2 (477.8 - 1060.7)	0.04	-152.8 (- 531.5 - 45.7)
p value	0.70	0.41		0.52

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, OPN - osteopontin.

Table 5-12 sTNFaR2 concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of sTNFaR2 (pg/ml)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	71.7 (34.5 - 101.8)	63.6 (50.9 - 125.8)	0.70	16.2 (-42.3 - 32.9)
Methacholine	50.6 (38.8 - 100.9)	59.9 (35.6 - 97.4)	0.75	9.7 (-30.9 - 36.1)
Saline	54.1 (27.7 - 83.9)	58.0 (43.4 - 65.4)	0.75	8.0 (-31.5 - 14.8)
Salbutamol / methacholine	53.2 (44.3 - 79.2)	42.5 (35.8 - 53.9)	0.04	-7.5 (-24.0 - 1.1)
p value	0.83	0.07		0.44

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, sTNFaR2 - soluble tumour necrosis factor alpha receptor 2.

Table 5-13 GROalpha concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of GroA (pg/ml)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	2323.5 (1652.3 - 2615.8)	3238.5 (2113.0 - 5751.8)	0.05	1024.6 (-322.3 - 3943.1)
Methacholine	2408.0 (1712.3 - 4157.0)	2994.0 (1953.0 - 4262.5)	0.81	-53.5 (-929.0 - 1364.3)
Saline	1955.5 (1439.0 - 2870.8)	3207.0 (1670.0 - 4742.0)	0.14	504.5 (-681.3 - 2932.5)
Salbutamol / methacholine	2171.5 (1783.3 - 2657.5)	2981.5 (1772.0 - 4374.5)	0.24	107.5 (-455.0 - 2166.0)
p value	0.90	0.93		0.61

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage.

Table 5-14 Activin A concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of Activin A (pg/ml)		p value for within group difference	Change (Δ) between challenges
	Pre challenge	Post challenge		
Allergen	0.0 (0.0 - 0.0)	209.5 (0.0 - 434.2)	0.03	209.5 (0.0 - 398.2)
Methacholine	0.0 (0.0 - 278.9)	264.8 (0.0 - 373.4)	0.16	0.0 (-18.5 - 373.3)
Saline	63.0 (0.0 - 254.7)	232.2 (33.7 - 338.8)	0.29	50.5 (-55.1 - 242.4)
Salbutamol / methacholine	200.3 (0.0 - 310.1)	116.9 (0.0 - 368.8)	0.77	0.0 (-107.3 - 88.4)
p value	0.13	0.98		0.33

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage

Table 5-15 Dipeptidyl peptidase 1 (DPP1) concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of DPP1 (ng/ml)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	123.3 (114.7 - 138.3)	132.6 (118.9 - 154.5)	0.21	16.5 (-2.4 - 31.2)
Methacholine	149.1 (124.3 - 168.5)	144.8 (125.5 - 171.2)	0.65	-11.9 (-35.8 - 18.3)
Saline	255.4 (124.7 - 332.7)	190.2 (135.7 - 255.6)	0.60	-90.9 (-179.7 - 130.9)
Salbutamol / methacholine	109.2 (88.6 - 142.4)	121.9 (105.7 - 171.9)	0.77	2.0 (-13.1 - 42.6)
p value	0.02	0.23		0.39

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, DPP1 - Dipeptidyl peptidase 1 (cathepsin C)

Table 5-16 Surfactant protein D (SPD) concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of SPD (ng/ml)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	367.0 (279.0 - 583.5)	532.0 (410.0 - 578.0)	0.23	195.0 (-188.0 - 272.0)
Methacholine	272.5 (156.5 - 436.3)	452.0 (363.0 - 576.0)	0.47	180.5 (-180.0 - 274.0)
Saline	520.0 (222.5 - 818.3)	461.0 (245.5 - 676.5)	0.59	-43.0 (-569.0 - 215.0)
p value	0.49	0.54		0.66

n=9 in allergen group, n=7 in methacholine group, n=6 in saline group. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, SPD - surfactant protein D

Table 5-17 Galectin 3 concentrations in bronchoalveolar lavage fluid before and after repeated inhaled challenges.

	BAL concentration of Gal3 (pg/ml)			
	Pre challenge	Post challenge	p value for within group difference	Change (Δ) between challenges
Allergen	95.5 (72.0 - 108.6)	152.0 (109.9 - 214.5)	0.007	53.2 (39.6 - 84.7)
Methacholine	88.6 (80.2 - 101.2)	157.5 (123.1 - 196.4)	0.07	70.7 (25.9 - 110.6)
p value	1.00	0.95		0.64

n=10 in allergen group, n=4 in methacholine group. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Mann-Whitney U test. BAL - bronchoalveolar lavage, Gal3 - Galectin 3

5.3.6 Serum measurements

Osteopontin was measured in the serum of volunteers before and after repeated inhalation challenges. There were no significant differences between or within the groups at any point (Table 5-18).

Table 5-18 Osteopontin concentrations in serum fluid before and after repeated inhaled challenges.

	BAL concentration of OPN serum (pg/ml)			
	Pre challenge	Post challenge	p within group	Change (Δ) between challenges
Allergen	13003 (10181 - 18829)	14525 (9079 - 19016)	0.58	948 (-1835 - 3722)
Methacholine	13805 (11422 - 18931)	16104 (11502 - 19911)	0.59	483.0 (-1650 - 4310)
Saline	19003 (13209 - 22860)	18096 (13678 - 31889)	0.39	2545.0 (-3372 - 14754)
Salbutamol / methacholine	12698 (8486 - 13723)	12154 (10445 - 17642)	0.33	2303 (-1363 - 3922)
p value	0.09	0.26		0.92

n=12 for all groups. Values are medians with IQR in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage, OPN - osteopontin.

5.3.7 Reverse transcriptase PCR results

Biopsies taken before and after repeated inhalation challenge were homogenised and the RNA extracted as previously described. RNA was then reverse transcribed to cDNA and the expression of various genes calculated using the quantitative reverse transcription PCR method (rtPCR). The expression of each gene of interest was normalised to the geometric mean of three housekeeping genes; GAPDH, UBC and A2. Relative expression of the genes of interest was then calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data, as detailed above.

Genes of interest investigated by rtPCR were resistin like molecule beta (RELM β), Collagen III (COL3A1), Collagen I (COL1A1), mucin 5AC (MUC5AC), Forkhead box A2 (FOXA2), alpha smooth muscle actin (ASMA) Tenascin (TNXB), transforming growth factor beta 1 (TGFB1), Osteopontin (SPP1), and a disintegrin and metalloprotease 33 (ADAM33).

Collagen III

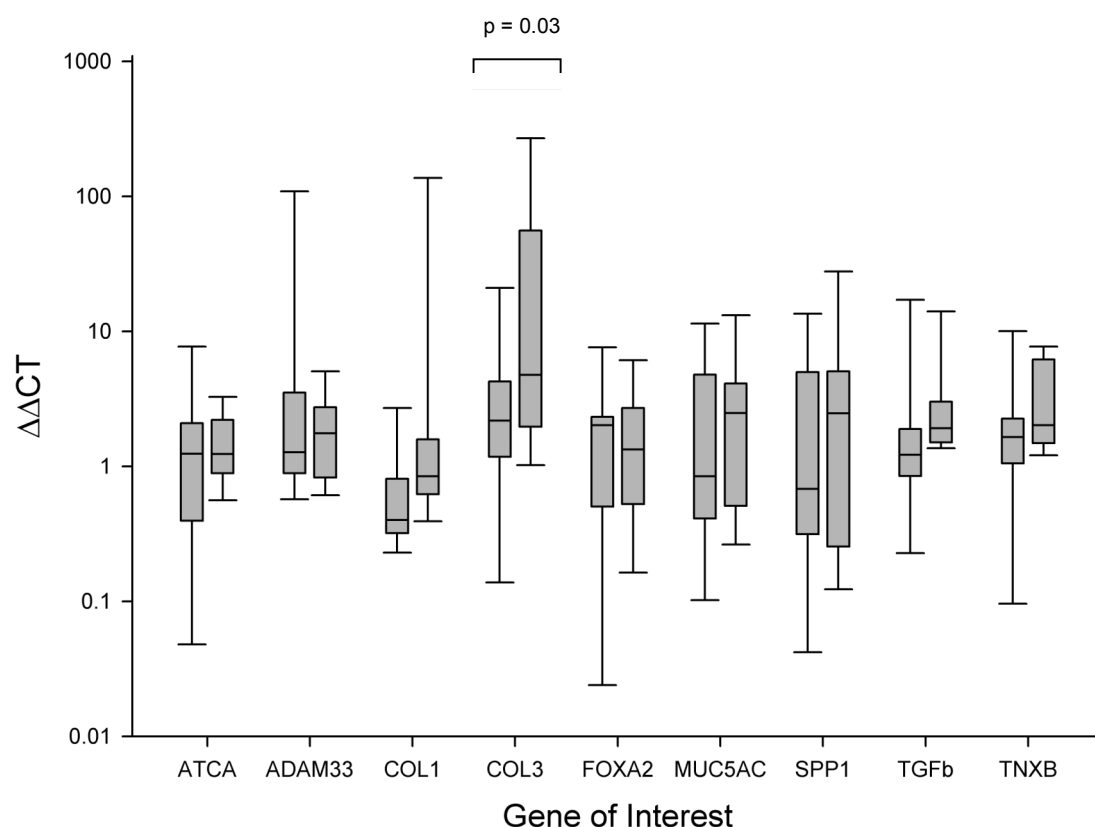
Collagen III gene expression was calculated as above in all exposure groups prior to and following repeated inhalation challenge. There was no difference in pre exposure expression; in the allergen group median (IQR) expression was 2.18 (1.17 - 4.26), in the methacholine group 1.71 (0.72 - 2.58), in the saline group 2.13 (0.52 (3.89) and in the salbutamol / methacholine group 0.51 (0.20 - 2.22) ($p=0.23$). There was a significant increase in the expression of collagen III in the allergen challenge group to 4.75 (1.96 - 55.78) ($p=0.03$) whilst there was an increase in the methacholine group which did not reach significance at 3.75 (1.54 - 6.68) ($p=0.08$). When the change in expression between the pre and post challenge biopsies was calculated the differences approached but did not achieve significance between the four groups ($p=0.07$) as shown in Figure 5-14, Figure 5-15, Figure 5-16 and Figure 5-17.

Table 5-19 Collagen 3 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	Collagen 3 relative expression		p within group	Change (Δ)
	Pre challenge	Post challenge		
Allergen	2.18 (1.17 - 4.26)	4.75 (1.96 - 55.78)	0.03	3.50 (-0.36 - 35.40)
Methacholine	1.71 (0.72 - 2.58)	3.75 (1.54 - 6.68)	0.08	1.61 (0.61 - 3.53)
Saline	2.13 (0.52 - 3.89)	2.17 (1.08 - 3.79)	0.58	0.12 (-0.29 - 0.95)
Salbutamol / methacholine	0.51 (0.20 - 2.22)	1.57 (0.54 - 3.00)	0.18	0.92 (0.14 - 2.82)
p value	0.23	0.04		0.07

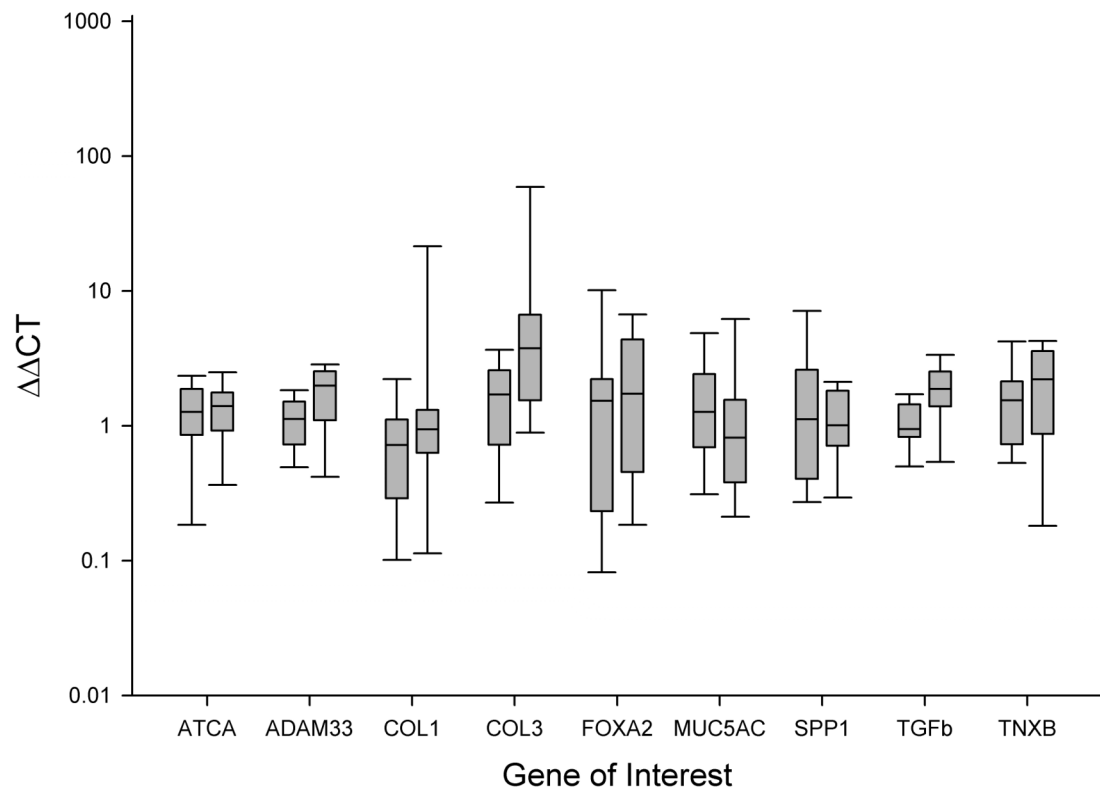
n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Figure 5-14 Relative expression (calculated by $\Delta\Delta CT$ method) of multiple genes of interest before and after repeated inhaled allergen challenge.



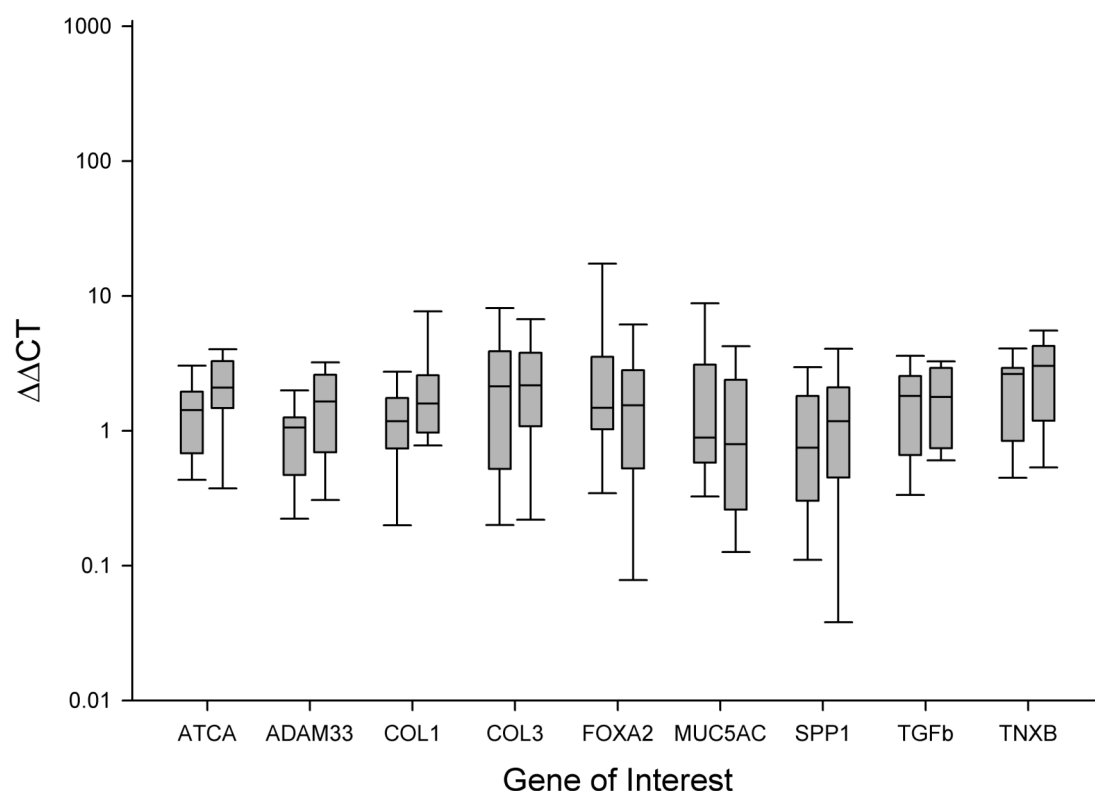
Left hand box plots are pre challenge, right hand box plots are post challenge. Genes of interest are; alpha smooth muscle actin (ATCA), a disintegrin and metalloprotease 33 (ADAM33), Collagen I (COL1A1), Collagen III (COL3A1), Forkhead box A2 (FOXA2), mucin 5AC (MUC5AC), Osteopontin (SPP1), transforming growth factor beta 1(TGFb) and Tenascin (TNXB). Gene expression is relative to arithmetic mean of pre-exposure ΔCT for each gene of interest. Differences are significant only where shown.

Figure 5-15 Relative expression (calculated by $\Delta\Delta CT$ method) of multiple genes of interest before and after repeated inhaled methacholine challenge.



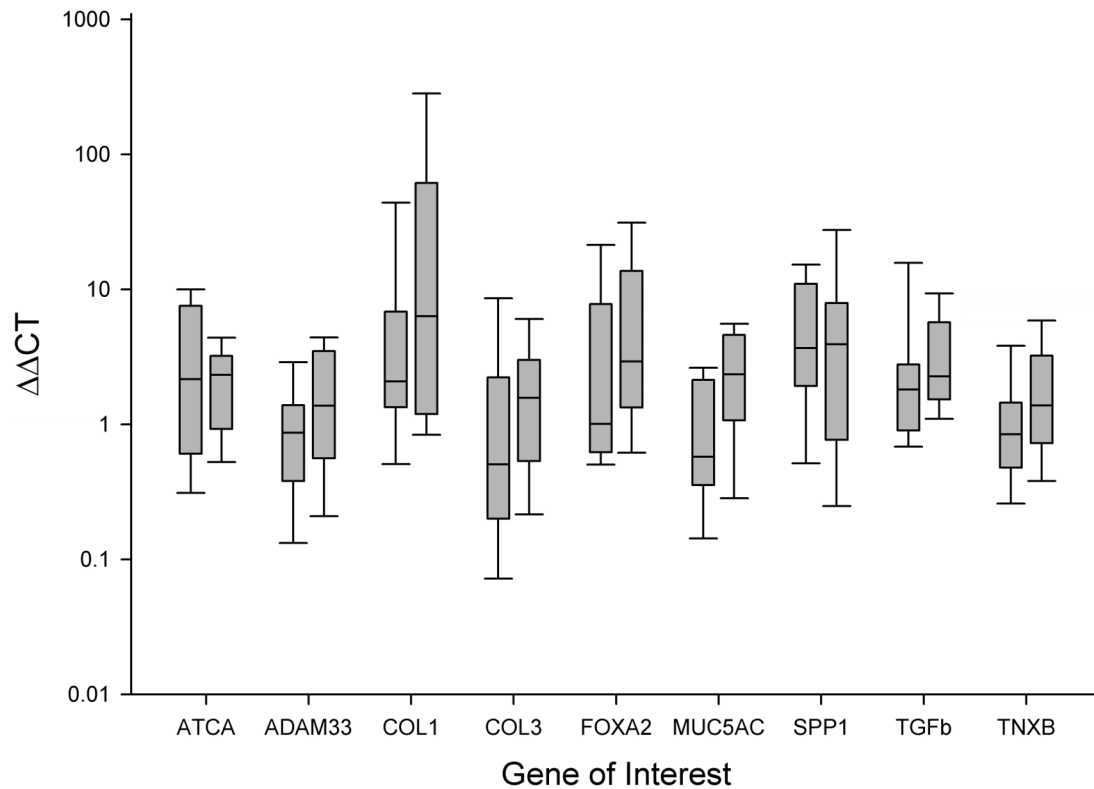
Left hand box plots are pre challenge, right hand box plots are post challenge. Genes of interest are; alpha smooth muscle actin (ATCA), a disintegrin and metalloprotease 33 (ADAM33), Collagen I (COL1A1), Collagen III (COL3A1), Forkhead box A2 (FOXA2), mucin 5AC (MUC5AC), Osteopontin (SPP1), transforming growth factor beta 1(TGFb) and Tenascin (TNXB). Gene expression is relative to arithmetic mean of pre-exposure ΔCT for each gene of interest. Differences are significant only where shown.

Figure 5-16 Relative expression (calculated by $\Delta\Delta CT$ method) of multiple genes of interest before and after repeated inhaled saline challenge.



Left hand box plots are pre challenge, right hand box plots are post challenge. Genes of interest are; alpha smooth muscle actin (ATCA), a disintegrin and metalloprotease 33 (ADAM33), Collagen I (COL1A1), Collagen III (COL3A1), Forkhead box A2 (FOXA2), mucin 5AC (MUC5AC), Osteopontin (SPP1), transforming growth factor beta 1(TGFb) and Tenascin (TNXB). Gene expression is relative to arithmetic mean of pre-exposure ΔCT for each gene of interest. Differences are significant only where shown.

Figure 5-17 Relative expression (calculated by $\Delta\Delta CT$ method) of multiple genes of interest before and after repeated inhaled salbutamol then methacholine challenge.



Left hand box plots are pre challenge, right hand box plots are post challenge. Genes of interest are; alpha smooth muscle actin (ATCA), a disintegrin and metalloprotease 33 (ADAM33), Collagen I (COL1A1), Collagen III (COL3A1), Forkhead box A2 (FOXA2), mucin 5AC (MUC5AC), Osteopontin (SPP1), transforming growth factor beta 1(TGFb) and Tenascin (TNXB). Gene expression is relative to arithmetic mean of pre-exposure ΔCT for each gene of interest. Differences are significant only where shown.

Other genes of interest

In no other genes of interest were there increases or decreases in the expression of the genes following the repeated inhalation challenges which were significant. The data are shown at Table 5-20, Table 5-21, Table 5-22, Table 5-23, Table 5-24, Table 5-25, Table 5-26 and Table 5-27 and also above in Figure 5-14, Figure 5-15, Figure 5-16 and Figure 5-17.

Table 5-20 Collagen 1 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	Collagen 1 relative expression			
	Pre challenge	Post challenge	p within group	Change (Δ)
Allergen	0.40 (0.32 - 0.81)	0.85 (0.62 - 1.58)	0.03	0.35 (0.13 - 1.34)
Methacholine	0.72 (0.29 - 1.11)	0.94 (0.63 - 1.31)	0.16	0.38 (-0.21 - 0.83)
Saline	1.18 (0.74 - 1.75)	1.58 (0.97 - 2.58)	0.08	0.37 (-0.06 - 1.05)
Salbutamol / methacholine	2.08 (1.34 - 6.84)	6.34 (1.19 - 61.31)	0.13	4.04 (-0.48 - 61.89)
p value	0.02	0.09		0.79

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-21 A disintegrin and metalloprotease 33 (ADAM33) relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	ADAM33 relative expression			
	Pre challenge	Post challenge	p within group	Change (Δ)
Allergen	1.27 (0.89 - 3.52)	1.76 (0.83 - 2.74)	0.35	0.20 (-0.10 - 0.55)
Methacholine	1.13 (0.73 - 1.51)	1.99 (1.10 - 2.54)	0.02	0.54 (0.02 - 1.34)
Saline			0.06	
Salbutamol / methacholine	0.87 (0.38 - 1.39)	1.38 (0.56 - 3.49)	0.14	0.67 (-0.43 - 2.18)
p value	0.13	0.92		0.38

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-22 Alpha smooth muscle actin (ASMA) relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	Alpha smooth muscle actin relative expression			
	Pre challenge	Post challenge	p within group	Change (Δ)
Allergen	1.24 (0.39 - 2.09)	1.23 (0.89 - 2.20)	1.00	-0.16 (-1.00 - 1.42)
Methacholine	1.26 (0.85 - 1.88)	1.40 (0.92 - 1.77)	0.64	-0.09 (-0.45 - 0.82)
Saline	1.42 (0.68 - 1.95)	2.09 (1.47 - 3.29)	0.04	1.02 (0.38 - 1.66)
Salbutamol / methacholine	2.16 (0.61 - 7.53)	2.32 (0.93 - 3.21)	0.35	-0.25 (-5.06 - 0.70)
p value	0.43	0.14		0.14

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-23 MUC5AC relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	MUC5AC relative expression		p within group	Change (Δ)
	Pre challenge	Post challenge		
Allergen	0.84 (0.41 - 4.77)	2.48 (0.51 - 4.11)	0.64	0.37 (-1.5 - 3.41)
Methacholine	1.27 (0.69 - 2.43)	0.82 (0.38 - 1.56)	0.27	-0.50 (-1.14 - 0.42)
Saline	0.89 (0.58 - 3.08)	0.80 (0.26 - 2.38)	0.64	-0.21 (-2.68 - 1.46)
Salbutamol / methacholine	0.57 (0.36 - 2.13)	2.34 (1.08 - 4.59)	0.05	0.82 (0.40 - 2.13)
p value	0.54	0.14		0.09

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-24 FOXA2 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	FOXA2 relative expression		p within group	Change (Δ)
	Pre challenge	Post challenge		
Allergen	2.02 (0.50 - 2.32)	1.33 (0.53 - 2.70)	0.53	-0.59 (-1.9 - 1.23)
Methacholine	1.53 (0.23 - 2.22)	1.73 (0.45 - 4.36)	0.53	0.11 (-0.56 - 0.99)
Saline	1.48 (1.02 - 3.53)	1.54 (0.53 - 2.81)	0.48	-0.13 (-0.89 - 0.65)
Salbutamol / methacholine	1.01 (0.62 - 7.80)	2.92 (1.34 - 13.69)	0.37	0.66 (-1.95 - 12.96)
p value	0.86	0.17		0.53

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-25 Tenascin relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	Tenascin relative expression		p within group	Change (Δ)
	Pre challenge	Post challenge		
Allergen	1.64 (1.05 - 2.26)	2.01 (1.49 - 6.18)	0.21	0.53 (-0.45 - 1.98)
Methacholine	1.54 (0.73 - 2.13)	2.21 (0.87 - 3.58)	0.18	0.34 (-0.28 - 1.19)
Saline	2.64 (0.84 - 2.92)	3.03 (1.19 - 4.26)	0.27	0.53 (-0.85 - 1.84)
Salbutamol / methacholine	0.84 (0.47 - 1.45)	0.38 (0.72 - 3.23)	0.27	0.32 (-0.36 - 2.42)
p value	0.17	0.42		1.00

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-26 SPP1 relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	SPP1 relative expression		p within group	Change (Δ)
	Pre challenge	Post challenge		
Allergen	0.68 (0.32 - 4.99)	2.47 (0.26 - 5.05)	0.94	-0.07 (-0.39 - 1.70)
Methacholine	1.12 (0.41 - 2.59)	1.01 (0.71 - 1.81)	0.58	0.04 (-0.81 - 0.28)
Saline	0.75 (0.31 - 1.81)	1.18 (0.45 - 2.09)	0.14	0.29 (-0.23 - 0.80)
Salbutamol / methacholine	3.67 (1.92 - 10.97)	0.91 (0.76 - 7.90)	0.24	-0.53 (-3.31 - 0.36)
p value	0.02	0.10		0.29

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

Table 5-27 TGF β relative gene expression in bronchial biopsies before and after repeated inhaled challenges.

	TGF β relative expression		p within group	Change (Δ)
	Pre challenge	Post challenge		
Allergen	1.22 (0.84 - 1.89)	1.91 (1.50 - 3.02)	0.06	0.60 (0.01 - 1.50)
Methacholine	0.95 (0.82 - 1.45)	1.88 (1.39 - 2.53)	0.006	0.78 (0.48 - 1.33)
Saline	1.81 (0.66 - 2.54)	1.78 (0.75 - 2.91)	0.94	0.13 (-0.92 - 0.74)
Salbutamol / methacholine	1.80 (0.90 - 2.78)	2.27 (1.54 - 5.71)	0.25	0.25 (-0.37 - 3.66)
p value between groups	0.19	0.45		0.28

n=12 for all groups. Values are median with interquartile range in parentheses. Within group comparison calculated using Wilcoxon test, between group differences calculated using Kruskal Wallis test. BAL - bronchoalveolar lavage. Relative expression of the genes of interest was calculated using the $\Delta\Delta C_T$ method using the arithmetic mean of the ΔC_T value for all pre-exposure samples to normalise the data.

5.3.8 Resistin like molecule beta (RELM β) in health and disease

As RELM β had not been examined previously in human airway disease, biopsies from healthy volunteers and from subjects with more severe asthma (regular inhaled steroids) were examined for percentage staining of RELM β and thickness of the basement membrane. Using thirteen healthy subjects, twenty two mild asthmatics selected at random from the pre-exposure bronchoscopies in the main study, and twenty two severe asthmatics whose characteristics can be seen in Appendix 4, there was a significant difference between both the thickness of the basement membrane and the percentage epithelial staining with an antibody to RELM β . In the healthy volunteers the median (IQR) percentage of the epithelium staining with RELM β was 1.89 (0.48 - 3.10)%, in the mild asthmatics 2.18 (1.05 - 4.01) and in the severe asthma group 5.89 (3.50 - 8.12) ($p < 0.001$). Using the Mann-Whitney test to compare between two groups, there was no difference between the healthy and mild asthma groups ($p = 0.24$) whilst there was a significant difference between the severe group and both the healthy and mild asthmatic subjects ($p < 0.001$) (Table 5-28 and Figure 5-18). Measurement of RELM β in the BAL from the same subjects gave similar results as in the challenge groups above, the majority of samples were below the limit of detection. The expression of the RELM β gene was assessed in brushings taken from similar groups of patients (though not the same) with 29 healthy subjects, 12 with mild asthma and 48 with severe asthma (clinical characteristics of these volunteers are shown in Appendix 5) the relative median (IQR) RELM β gene expression in the epithelial brushings was 12.4 (5.1 - 21.3) in the healthy subjects, 11.7 (5.4 - 64.4) in the mild asthmatics and 15.9 (8.3 - 33.6) in the severe asthmatics ($p = 0.33$) as shown in Table 5-29. RELM β mRNA could not be detected in primary human fibroblasts from normal or asthmatic donors either at rest or following stimulation with IL4 or IL13 (data not shown).

Table 5-28 Differences in immunoexpression of epithelial RELM β and collagenous basement membrane thickness in bronchial biopsies from healthy volunteers and subjects with mild and severe asthma.

	Age	BAL eosins	Collagen thickness (um)	RELM β % epithelium
Healthy (n=13)	30.6 (15.3)	0.0 (0.0 - 0.0)	4.39 (4.20 - 5.49)	1.89 (0.48 - 3.10)
Mild asthma (n=22)	26.2 (8.7)	1.25 (0.95 - 3.43)	7.47 (6.39 - 8.63)	2.18 (1.05 - 4.01)
Severe asthma (n=22)	48.2 (15.6)	0.8 (0.30 - 2.50)	6.36 (5.43 - 7.10)	5.89 (3.50 - 8.12)
p value	<0.001	<0.001	<0.001	<0.001

Values are median with IQR in parenthesis except age which is mean (SD). Between group differences calculated using Kruskal Wallis test except for age which was calculated using one way analysis of variance. Comparing between pairs of groups using the Mann-Whitney Test for RELM β percentage staining of the epithelium; healthy vs mild asthma $p=0.24$, healthy vs severe $p<0.001$, mild vs severe $p<0.001$. Comparing between pairs for BAL eosinophils; healthy vs mild asthma $p<0.001$, healthy vs severe asthma $p=0.002$, mild vs severe $p=0.16$. Comparing between pairs for collagen basement membrane thickness healthy vs mild $p<0.001$, healthy vs severe asthma $p=0.004$, mild vs severe $p=0.005$.

Figure 5-18 Percentage total epithelium immunoexpressing resistin like molecule beta (RELM β) in bronchial biopsies from healthy volunteers and subjects with mild and severe asthma.

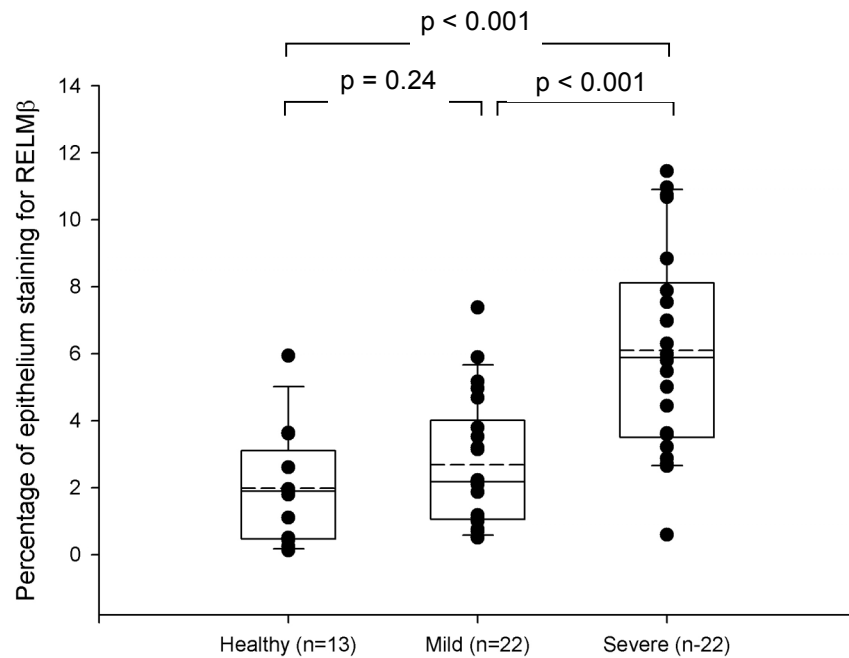


Table 5-29 Relative expression of RELM β mRNA in bronchial epithelial brushings from healthy volunteers and subjects with mild and severe asthma.

	RELM β relative gene expression ($\Delta\Delta$ CT)
Healthy (n=29)	12.4 (5.1 - 21.3)
Mild asthma (n=17)	11.7 (5.4 - 64.4)
Severe asthma (n=55)	15.9 (8.3 -33.6)
p value	0.33

Values were calculated by $\Delta\Delta$ CT method using UBC and GAPDH as housekeeping genes, and are expressed as median with IQR in parenthesis. p value calculated using Kruskal Wallis test.

5.4 Discussion

This work has demonstrated for the first time that airway remodelling may be induced by bronchoconstriction in the absence of an increase in eosinophilic inflammation.

5.4.1 Epithelial activation following allergen and methacholine challenges

Epithelial activation with increased TGF β and RELM β staining were seen following both repeated allergen and methacholine challenges. These findings support multiple cell culture models, which suggest that mechanical stress on the airway results in epithelial activation, including the release of active TGF β from intracellular stores (Tschumperlin 2003, Park 2009). Interestingly the increase in TGF β expression in the epithelium was greater in the methacholine group compared to the allergen group, despite the absence of the late allergic response and therefore less bronchoconstriction. It is possible that the inflammatory response seen in the allergen group, with the increase in eosinophils found in the BAL, if not the tissue, resulted in increased submucosal TGF β production. If tissue eosinophils were a source of TGF β , as they have been shown to be in the past (Flood-Page 2003) they may have reduced TGF β production within the epithelium by a negative feedback loop. This process would have been absent in the methacholine group, and hence the greater epithelial TGF β response seen. This would raise the intriguing possibility that reducing airway eosinophils might increase the production of TGF β by airway epithelial cells in response to bronchoconstriction, though this is pure speculation it could be assessed *in vitro* using a cell compression system with and without the presence of eosinophils.

For the first time in humans, a novel potential mediator of airway remodelling, RELM β , was investigated both in health and disease and following allergen and

methacholine challenge. In keeping with the TGF β response, RELM β protein expression was increased following both allergen and methacholine challenges within the exposure groups, though there was no significant between group differences when compared to saline or salbutamol / methacholine control groups. The activation pathway for RELM β is very likely to include STAT6, and there is animal evidence that is also IL13 mediated. IL13 is a characteristic Th2 cytokine, one that would be expected to be elevated following allergen challenge, but not following the purportedly immunologically inert methacholine challenge. Although there has been no work examining IL13 activation following mechanical stress within the airway, IL13 has been shown to be upregulated following mechanical stress in cardiomyocytes (Nishimura 2008). It is possible that IL13 is upregulated in epithelial cells following compressive stress, and this would provide a mechanism by which 'Th2' cytokines might be upregulated in the absence of Th2 cells. In order to investigate this further, the expression of IL13 or other Th2 cytokines could be examined in tissue sections by immunohistochemistry, or by qPCR analysis, and in the BAL by ELISA. Alternatively the expression of Th2 cytokines could be examined *in vitro*, though this has not been done, recent work has shown that other inflammatory cytokines including the chitinase-like protein YKL-40 is increased following bronchial epithelial cell compression (Park 2010).

Using immunohistochemistry to investigate any protein depends on the specificity of binding of the antibody available; with a novel protein such as RELM β there is the additional problem that few antibodies exist, and they have been subjected to less use and review than with well established antibodies. It is possible that the antibody we used is not binding to RELM β , rather to some other antigen which is upregulated following allergen challenge. Isotype controls of the immunohistochemical staining with RELM β antibody were negative, as were preabsorption experiments using recombinant protein (as shown above), however there still remains the possibility that the antibody is not binding to RELM β . In the absence of a spontaneous human knockout, these are probably

the best available controls for the antibody binding specificity. The low levels or negative detection of RELM β in the BAL fluid either before or after inhalation challenge or in the healthy or asthmatic volunteers does not mean that RELM β is not present in the tissue or important in asthma pathogenesis. TGF β is accepted to be important in asthma, and is increased in the tissue and BAL of asthmatics, though the BAL has to be concentrated in most circumstances in order to bring the levels into the range of detection of most ELISAs. The biologically active concentration of TGF β is well below the concentration detectable by ELISA (10 fM, 0.4 pg/ml) (Olsson 2000) as is the mitogenic effect of RELM β on human fibroblasts which is detectable at 0.5 nM (4.5 pg/ml) (Mishra 2007). It is possible that concentration of the BAL fluid would enable measurement of the RELM β concentration using the available ELISA, this has not been performed so far, as this would limit significantly the BAL available for other assays.

5.4.2 Threshold for response to bronchoconstriction?

In the allergen challenge and methacholine challenge groups the early bronchoconstrictive responses were well matched, although there was no late allergic reaction in the methacholine group. This would imply that there was more bronchoconstriction in the allergen group, though the exact makeup and cause of the fall in FEV₁ during the LAR is not fully understood and may not be entirely due to airway smooth muscle constriction, but may also have elements of airway oedema and mucus secretion leading to a decrease in the FEV₁. However, it is likely that there was a greater degree of bronchoconstriction in the allergen group, so why was the degree of airway remodelling not significantly different in the allergen and methacholine groups? The study was not powered for equivalence, and no claim for equivalence is made; it is unlikely that such a study would ever be performed due to the numbers of individuals required, however the lack of difference should be examined. It is possible that there is a threshold level of bronchoconstriction above which remodelling will be triggered, and once triggered any further bronchoconstriction will not lead to

additional remodelling. Using *in vitro* models of physical stress mimicking remodelling it has been shown that compressive stress on the epithelium for 10 minutes a day induced a similar degree of remodelling (as measured by mucus cell hypertrophy and hyperplasia *in vitro*) as 1 hour daily (Park 2009). These findings are in keeping with results that show that at a molecular level the response to compressive stress is transient and complete within minutes (Tscumperlin 2002, 2004), it appears that it is the change in stress that induces the response, rather than the compressive stress itself. This could explain why in the allergen challenge group, where there was a longer duration of bronchoconstriction, there was a similar degree of airway remodelling. It is of course possible that there was a difference in the degree or nature of the airway remodelling induced by allergen compared to methacholine, however the current study was not powerful enough to detect that difference. This is considered unlikely as there was no trend towards this finding, and in some measures, such as the TGF β in the epithelium, there was actually a greater change following methacholine challenge.

5.4.3 Role of eosinophils in airway remodelling

If the increase in eosinophils in the allergen challenge group does not appear necessary for the induction of remodelling, why is it that eosinophil depletion studies show a reduction in the markers of airway remodelling? It has been demonstrated that reduction of eosinophils using anti IL5 antibodies causes a reduction in exacerbations of asthma, and an improvement in asthma related quality of life (Halder 2009, Nair 2009). If the reduction in eosinophils due to anti L5 treatment lead to a decrease in exacerbations, then as the exacerbations (and indeed many of the symptoms of asthma) are caused by bronchoconstriction, the reduction in eosinophils would lead to a reduction of bronchoconstriction, and so to a reduction in airway remodelling were bronchoconstriction the direct cause. The reduction in eosinophil numbers would be associated with a reduction in airway remodelling, but would be acting

via an intermediate step of reduction in bronchoconstriction being the attributable cause.

5.4.4 Airway permeability changes following challenges

It has been previously demonstrated that airway permeability increases after single segmental allergen challenge induced at bronchoscopy when measured by radio labelled serum proteins. Levels of BAL proteins increase by 2-4 hours following aerosol challenge, though numbers in this initial study performed in 1987 were small (n=4) (Fick 1987). $\alpha 2$ macroglobulin, a 725kd plasma protein, has also been shown to be increased in BAL fluid following segmental allergen challenge only 10 minutes after challenge. In the same study by Svensson *et al*, nasal allergen challenge was performed which also induced an increase in $\alpha 2$ macroglobulin concentrations in nasal lavage fluid (Svensson 1995). This extremely rapid increase in $\alpha 2$ macroglobulin was not found in a similar study also using segmental allergen challenge delivered by bronchoscope. In this study performed by Nocker *et al*, plasma leakage, measured by $\alpha 2$ macroglobulin and albumin concentrations, was measured 5 minutes and 4 hours following challenge. There was a trend towards an increase in $\alpha 2$ macroglobulin at 5 minutes, with a significant increase by 4 hours (Nocker 1999). The dose of allergen used by Svensson *et al* was greater than that used by Nocker *et al*, which could explain the different results obtained.

In light of these previous data, a rise in $\alpha 2$ macroglobulin in our allergen challenge group was not unexpected, though for such a rise to be sufficiently prolonged to show a significant difference 4 days after the last challenge is somewhat surprising. The effect on the passage of plasma proteins across the respiratory epithelium following methacholine challenge has been less extensively studied than following allergen challenge. Most studies have been performed measuring $\alpha 2$ macroglobulin concentrations in induced sputum following inhaled challenges, although some have been performed on nasal

lavage. Halldorsdottir *et al* examined the concentrations of $\alpha 2$ macroglobulin in sputum obtained from healthy volunteers 5 minutes after inhalation challenge with histamine, capsaicin or methacholine. In her 'healthy' subjects, methacholine induced a mean 18.4% drop in FEV₁ which brings into doubt their clinical characterisation. There was an increase in $\alpha 2$ macroglobulin following both the histamine and methacholine challenges (Halldorsdottir 1997). Nasal provocation tests with normal saline and methacholine showed an increase in albumin, total protein, secretory IgA and total IgA in nasal lavage following methacholine, but not saline challenge (Raphael 1988). Not all studies have found an increase in airway permeability following methacholine challenge, Belda *et al* looked at mild asthmatics subjected to adenosine and methacholine challenges matched to a 20% fall in FEV₁, and measured $\alpha 2$ macroglobulin, albumin and differential cell counts in induced sputum 48 to 72 hours after a single challenge. There was a small increase in albumin in sputum, but no change in $\alpha 2$ macroglobulin following methacholine challenge. The authors concluded that adenosine but not methacholine induces mild airway plasma exudation, though the study was flawed in its experimental design, with no true control group being used (Belda 2005). In healthy people Grieff *et al* measured fucose (a marker of mucin secretion) and $\alpha 2$ macroglobulin in nasal lavage 10 minutes after nasal histamine, methacholine or capsaisin challenges. They noted an increase in fucose and $\alpha 2$ macroglobulin following histamine challenge, but only an increase in fucose following methacholine challenge (Grieff 2005).

A possible mechanism by which methacholine might increase the permeability of the airway to $\alpha 2$ macroglobulin would be by the activation of the epithelium by mechanical stress as previously described; such activation may interfere with the function of tight junctions. Such tight junction breakdown has been seen in some *in vitro* models of bronchoconstriction, resulting in more rapid transit of lentivirus deep to the epithelium (Tomei 2008), but not in others when measured by transit of FITC labelled dextran across the epithelial surface (Park

2010). It may be that repeated challenge induces a greater breakdown in tight junctions than a single challenge, hence the more substantial increase in $\alpha 2$ macroglobulin concentrations in our *in vivo* study compared to others. In addition BAL studies may be more sensitive than those on induced sputum. It is unlikely that repeated bronchoscopy or repeated spirometry induced the change in airway permeability as no such increase was seen in the groups exposed to saline or salbutamol / methacholine challenge.

5.4.5 Why no qPCR results?

The almost complete lack of positive results from the qPCR investigations was disappointing, but not entirely unexpected. Previous examination of the change in regulation of gene expression following bronchoconstriction has not been performed *in vivo*, however has been performed *in vitro*. In the *in vitro* studies, using much earlier time points than in our study, the change in regulation of genes in response to compressive stress appears either absent, suggesting post transcriptional adaptation pathways, or rapid and transient. TGF β 1 and 2 and endothelin 1 and 2, examined in human bronchial epithelial cells (HBEC's) following a single compressive episode *in vitro* showed that the endothelin 1 gene was upregulated significantly at 4 and 8 hours and still upregulated at 24 hours following compression, whilst endothelin 2 was upregulated at 4 and 8 hours, but by 24 hours expression levels were the same as baseline. There was no change in either TGF β gene at any time point (Tschumperlin 2003). Other genes appear even more rapid in their response to stress, HBEGF and epiregulin are significantly upregulated after a single compression *in vitro* after 1 and 4 hours after compression, but their expression is back to baseline at 8 hours (Chu 2005). Our model used repeated compression, and it may have been hypothesised that such repeated compressive or mechanical stress might result in more sustained regulation changes in genes of interest, however this appears not to be the case. Repeated compression of HBEC's *in vitro* for 14 days lead to no significant change in FOXA2 or MUC5AC expression when measured 24 hours after the last compression; if the genes were measured 24

hours after a single compression stress the authors of the study reported a statistically significant reduction in FOXA2 expression, though the change in the $\Delta\Delta CT$ was only 0.8 using only 2 cell donors, and therefore of dubious experimental significance (Park 2009). Therefore the lack of significant results seen in the qPCR data was most likely due to timing of the sampling, any changes in gene expression were probably missed during the time between the last exposure and the second bronchoscopy. Despite this delay in sampling, there was still significant within group upregulation of collagen III expression in the allergen group and a trend towards an increase in the methacholine group, this is surprising given the *in vitro* data above, but may suggest that the stimulus to remodelling provided by inhalation challenge with allergen or methacholine triggers a longer term remodelling response than would have been expected by the cell models.

Another possibility is that the method used to detect a difference between the pre exposure and post exposure groups was insufficiently sensitive. As multiple housekeeping genes were used it is possible to estimate the minimum detectable difference in expression of genes of interest that could be detected using this system with the formula;

$$\text{Minimum detectable difference} = \frac{\left(\frac{\text{Conc}_{\text{House keeping gene 1}}}{\text{Conc}_{\text{House keeping gene 2}}} \right)_{\text{pre exposure}}}{\left(\frac{\text{Conc}_{\text{house keeping gene 1}}}{\text{Conc}_{\text{house keeping gene 2}}} \right)_{\text{post exposure}}}$$

If the normalised concentration for the pre exposure group were to equal the normalised concentration for the post exposure group, the minimum detectable difference (as a ratio) would be one fold, i.e. a doubling or halving of the expression between pre and post exposure groups would be detectable. Using the formula, the minimal detectable difference was calculated as 1.007 for the data presented above, suggesting that the lack of significant results was not due to insensitivity of the technique used.

In retrospect it would have been worth taking epithelial brushings for RNA extraction in order to separate out any epithelial response to the inhalation challenges compared to the mixed tissue in the bronchial biopsies. An alternative approach would be to use laser dissection of frozen biopsies that were taken at the time to examine the epithelial compartment. Such work was not done in this case as the negative results in the whole biopsies would suggest this approach would also be negative, though would be worth bearing in mind for future work.

5.4.6 Limitations of the current study

This preliminary study was performed with relatively small numbers of individuals, 12 in each group having repeated bronchoscopy. Such small numbers, although sufficient to demonstrate within and between group differences in markers of airway activation and remodelling may have been insufficient to detect changes between the groups for small differences. For example, there was a trend towards a lesser increase in the TGF β staining in the allergen group compared to the methacholine group. In order to establish whether this was a real difference with 80% power and a significance level of 0.05, 50 volunteers would be required in each group; such numbers are unlikely to be achieved with such an intensive and invasive study protocol.

Multiple different parameters were examined on the same sample set, and this raises the probability of obtaining a false positive or Type I error due to repeated analysis. Comparing between the groups using difference between the challenges reduces some of the repeated analysis compared to that which would have occurred if using comparison within groups, but this remains a problem. The standard statistical response to multiple comparisons on a single set of data is to perform a Bonferroni correction. This technique corrects for the testing of multiple independent hypotheses on a single set of data by testing each individual hypothesis at a significance level of the accepted significance level for a single hypothesis divided by the number of individual tests being performed. In the standard situation where the significance level is 0.05, then the calculation would be $0.05/n$ where n is the number of hypotheses being tested. Such a correction would reduce the probability of a Type I error, *i.e.* the rejection of the null hypothesis when it should not have been, but, especially with such a small sample size, would have raised the possibility of a Type II error, the failure to observe a difference, when in truth there is one present. In our study there was a single hypothesis being tested using a number of tested markers of that hypothesis, it has been argued that the use of a Bonferroni correction in such circumstances, by taking the total number of tested markers into account is essentially the same as testing the universal null hypothesis that none of the markers is associated with the remodelling process (Boehringer 2000). In our study the individual disease markers are of interest in their own right, rather than all of them in concert being examined as a block using the universal null hypothesis. By not performing a Bonferroni correction each marker of remodelling has been considered as an individual null hypothesis that is potentially involved in the aetiology of remodelling in its own right. This is really only correct, however where the markers are truly independent, as would be in unlinked genetic studies, therefore this is a compromise based on balancing the risk of Type I and Type II errors. In order to be more confident in the results more individuals should be recruited for a repeated study with the

same protocol, and power calculations performed as to the required numbers to assess the results following a Bonferroni correction.

5.4.7 A new asthma paradigm?

If bronchoconstriction leading to remodelling in the absence of additional airway inflammation is to be considered as a new asthma paradigm, then this new paradigm should be biologically plausible, have complementary animal and ideally complementary human evidence. The biological plausibility has been addressed with the *in vitro* work primarily performed by Tschumperlin and colleagues (Tschumperlin 2002, Park 2010), but with notable contributions from others including Swartz and colleagues (Swartz 2001, Tomei 2008). These groups have demonstrated that compression of human bronchial epithelial cells (HBEC's) *in vitro* leads, probably via shrinkage of the intercellular space around those cells, to autocrine binding of epidermal growth factor receptor ligands (Tschumperlin 2004). Compressing epithelial cells in air liquid interface (ALI) culture results in the cells staying same size, but fluid leaving the intercellular space (about 90% of the fluid was removed from the intercellular space by compression whilst the cell volumes themselves were relatively constant). The epithelial cells express the epidermal growth factor receptor (EGFR) ErbB1 and compressive stress *in vitro* leads to the matrix metalloproteinase dependent shedding of heparin binding epidermal growth factor (HBEGF); This then binds to the EGFR, resulting in its phosphorylation. By this mechanism there is no need for force dependent biochemical processes within the cell or cell membrane (Tschumperlin 2004). This mechanism also appears relevant in whole, though isolated airways; administration of methacholine to isolated mouse trachea leads to phosphorylation of the EGFR, this result was only found when mechanical stress occurred, as this was abrogated by pre administration of isoproterenol (Tschumperlin 2004).

If a new paradigm is to be established, the evidence should ideally come from more than one group, using more than one technique. Tschumperlin and colleagues use a system of increasing pressure over HBEC's *in vitro* to mimic the effects of bronchoconstriction, whereas Swartz *et al* have developed a complementary technique. In this method, a three dimensional *in vitro* model of the airway has been developed where human fibroblasts are suspended in a collagen matrix with differentiated human bronchial epithelial cells grown on the surface of that matrix (Choe 2003). Compressive stress is then applied to the whole matrix laterally (rather than apically) to mimic compressive stresses within the airway during bronchoconstriction. Using this coculture airway model they have shown that compressive stress leads to increased fibroblast deposition of collagens I, III and V in the absence of an inflammatory infiltrate (Choe 2006). In addition, the collagen deposition was greatest near to the epithelium, and decreased according to distance, suggesting epithelial control of the remodelling process. This technique has not been used to examine other elements of airway remodelling such as epithelial cell metaplasia, though the apical pressure technique has demonstrated that the repeated mechanical stress of HBEC's *in vitro* leads to increased mucus production and epithelial metaplasia to a mucus secreting phenotype (Park 2009).

There are therefore multiple lines of evidence suggesting *in vitro* that the compressive stress associated with bronchoconstriction leads to remodelling processes. If this remains the case *in vivo*, then the new paradigm suggested here would be further supported. Using a guinea pig model of asthma, Gosens *et al* administered OVA once weekly for 12 weeks, and gave tiotropium (a long acting anticholinergic therapy (reviewed in Bateman 2009)) 30 minutes prior to OVA administration. The OVA induced an increase in alpha smooth muscle actin (ASMA) and airway smooth muscle (ASM) mass in the smaller airways, which was attenuated by the tiotropium (Gosens 2005). Notably the tiotropium only changed the ASMA and ASM mass in the animals exposed to OVA; the authors implied that this was due to the effects of endogenous acetylcholine in

combination with eosinophilic inflammation inducing remodelling, that was then blocked by the tiotropium. An alternative explanation, not put forward by the authors, is that the tiotropium reduced airway bronchoconstriction (which was demonstrated in isolated tracheal rings exposed to OVA) during the OVA challenges, and hence reduced the degree of remodelling that took place.

This animal model is indirectly supportive of our hypothesis; if this were correct, then it would be expected to find other supporting data to suggest that tiotropium or other bronchodilators would reduce remodelling in other situations. The most commonly used bronchodilators in asthma are the β_2 agonists, either in short acting (short acting beta agonist (SABA)) or long acting beta agonist (LABA) form. There have been few studies examining the effects of β_2 agonists on airway remodelling directly, though some have been performed. Altraja and colleagues performed a study in which asthmatics were bronchoscoped prior to being given four times daily salbutamol (a SABA) or nedocromil sodium (not a bronchodilator, a steroid sparing agent which stabilises mast cells) for twelve weeks, prior to being bronchoscoped again. They found a reduction in the mean thickness of subepithelial tenascin thickness between the two bronchoscopies in the salbutamol, but not the nedocromil groups (Altraja 1999). This finding was attributed to the anti-inflammatory effects of salbutamol resulting in decreased remodelling in the airway. Studies on LABA's have shown similar results, in a study examining the vascularity of the airway as a marker of the remodelling process it has been shown that increasing the dose of inhaled corticosteroid had no effect on airway vascularity, whilst adding a LABA lead to a significant (approximately 20%) reduction in airway vascularity (Orsida 2001), and also a reduction in BAL IL8 concentration (Reid 2003). The authors proposed that these effects were due to synergistic effects of the LABA and the inhaled corticosteroid reducing inflammation, rather than the alternative explanation that decreased bronchoconstriction lead to decreased epithelial activation and therefore remodelling. These findings with LABA's are not universal however, Lindqvist *et al* showed no effect of administration of a LABA on subepithelial

tenascin or eosinophilic inflammation, and although inhaled corticosteroids in this study reduced tissue eosinophils, there was also no reduction in subepithelial tenascin (Linqvist 2003). The different findings in these studies may be due to the populations studied, in the study by Orsida and colleagues, the volunteers were all uncontrolled asthmatics, which by definition means asthmatics that are having episodes of symptoms due to bronchoconstriction, whereas in the study by Linqvist *et al*, the asthmatics were described as 'newly diagnosed'. In addition Lindqvist and colleagues induced bronchoconstriction in all the groups three days prior to their second bronchoscopy to assess AHR; if bronchoconstriction induces airway remodelling this may have been sufficient to confound the results.

In a study performed by Kelly *et al* (Kelly 2010) a single group of subjects with mild asthma underwent allergen challenge following 11 days of treatment with either placebo, inhaled budesonide (Pulmicort Turbohaler 200µg bd) or combined long acting beta agonist (LABA) and budesonide (Symbicort Turbohaler; budesonide 200µg and formoterol 6µg twice daily). The group were then bronchosoped with biopsies taken 24 hours after the allergen challenge. The Symbicort group had a significantly reduced early (EAR) and late (LAR) falls in FEV₁ following allergen challenge; the bronchconstrictive response to the allergen was unchanged in the placebo or budesonide groups. The eosinophil response measured using induced sputum was no different following allergen challenge between the 3 groups, though there was a within group reduction in the number of eosinophils in the Symbicort group. As a marker of remodelling, the number of myofibroblasts in bronchial biopsies was measured. In the placebo group there was a large and statistically significant increase in the numbers of myofibroblasts, and this increase was similar in the budesonide group, but the LABA / corticosteroid group showed a marked reduction in the numbers of myofibroblasts following allergen challenge, approaching baseline.

Therefore in this study there was reduced airway constriction measured by reduced EAR and LAR following allergen challenge, associated with a reduction in the airway myofibroblast response to allergen challenge. Though there was a within group reduction in eosinophils in the LABA / steroid group there were no between group differences in eosinophilic inflammation, whilst there were significant between group differences in the myofibroblast numbers and the degree of bronchoconstriction in the allergen challenges.

The authors concluded that the effects on the bronchoconstriction following the allergen challenge are due to the direct LABA effects on the smooth muscle in the airway (Kelly 2010). The effects of the LABA on the remodelling was, however, attributed by the investigators to either "budesonide and formoterol activate mechanistically distinct anti-inflammatory pathways that combine to produce an additive clinical response" or "formoterol augments the activity of budesonide through a common mechanism or mechanisms to a degree of synergistic anti-inflammatory response". They go on to state that they believe the second scenario to be more likely; that the LABA augments the anti-inflammatory effects of the budesonide, so reducing the airway remodelling. In view of our results in this study, it is possible, maybe even likely, that the reduction in airway remodelling in the LABA / steroid group was due, not to an augmentation of the steroid effect on inflammation, but rather to the documented reduction in bronchoconstriction seen during the allergen challenge with the LABA.

This study was notably confounded by the addition of corticosteroids with the LABA. Our study has not demonstrated that the eosinophils present following allergen challenge do not induce airway remodelling but we have shown that bronchoconstriction in the presence of underlying eosinophilic inflammation, but not an increase in eosinophils, does induce airway remodelling. To investigate this further a study examining the effects of blocking bronchoconstriction following allergen challenge similar to the Kelly study, but with perhaps a

combination of short and long acting beta agonists and no corticosteroids would be useful. This would examine the effect that eosinophilic inflammation alone had on airway remodelling in the absence of bronchoconstriction. Another way to study this would be treat subjects with the humanised mouse monoclonal antibody against IL5 (Mepolizumab) which has been shown to decrease both circulating and tissue eosinophils, and then induce bronchoconstriction using methacholine. This would address one of the remaining questions from our study, *i.e.* that that we have induced bronchoconstriction in the presence of eosinophils and demonstrated that a rise in eosinophils numbers is not required in order to induce remodelling, but we have not shown that eosinophils are not required *per se*, merely that an increase in eosinophil numbers is not required. It may be that bronchoconstriction alone is insufficient to induce remodelling *in vivo*, and that the presence of eosinophils in the airways is required. By treating with Mepolizumab and then inducing bronchoconstriction this possibility could be investigated. Also it would be interesting to investigate the mechanical stress induced in the airway during bronchoconstriction on eosinophils to examine whether mechanical stress can induce eosinophil activation, even if it does not appear to induce increased eosinophil numbers. Currently no investigations of this manner have been reported.

5.4.8 Remodelling, a normal response to mechanical stress?

Our data raise the possibility that remodelling is not a primary abnormality in asthma at all, rather that remodelling is a normal response to bronchoconstriction. A situation could be envisaged where physical stress on an individual cell leads to that cell inducing the production of cytokines which increase the stability and thickness of its underlying substrate, in order to decrease the effects of the mechanical stress. This process would be relevant to all cells in the airway (and indeed many other tissues where this has been shown to be the case) no matter whether the individual was asthmatic or not. The cells in the airways of asthmatics would be the subject to the mechanical stress of bronchoconstriction, whereas those in the airways of normal

individuals would only be exposed to the mechanical stress associated with tidal breathing. It is possible that the airway remodelling seen in asthma could be a normal response to the physical stress caused by bronchoconstriction, *i.e.* normal individuals would undergo airway remodelling if you were able to make them bronchoconstrict. The remodelling response would then be a normal response to an abnormal event and as that abnormal event only happens in asthmatics, the remodelling is only seen in the asthmatics. There is a second possibility, that asthmatics respond more to any bronchoconstriction stimulus than normal individuals, *i.e.* they have an accentuated response to the abnormal stimulus. In addition, there is a final possibility, that the response to bronchoconstriction seen in asthmatics is unique to the asthmatic population and the airways of normal individuals are resistant to a physical stimulus inducing change. This final possibility is unlikely as physical stress is a requirement for normal lung development (Mollard 1998, Moore 2005) and the work mentioned above by Swartz and Tschumperlin has been performed on normal HBEC's, rather than those from asthmatics. As it is therefore unlikely that there is a completely unique pathway of response to mechanical stress in the asthmatic population that we studied; the other two possibilities remain, that the response to bronchoconstriction we saw was a normal response to such physical stress, or that the response of the asthmatic airway is different for a certain degree of mechanical stress than the normal airway. If the second possibility were correct, this would imply that there are two sequential abnormalities in the asthmatic airway, that of having bronchoconstriction in the first place, and then an abnormal or exaggerated response to it. In order to investigate the hypothesis that arises from these questions, that normal individuals and asthmatics respond the same to bronchoconstriction, the study must switch to *in vitro* modelling as it is impossible to bronchoconstrict normal individuals to the same degree as asthmatics, using methacholine or other inhaled agents. In order to address this question, a model of epithelial bronchoconstriction *in vitro* was established as discussed in the next chapter.

Chapter 6 *in vitro* modelling of bronchoconstriction

The previous chapters have demonstrated that remodelling in asthma is independent of additional eosinophilic inflammation and dependent on bronchoconstriction as a stimulus to remodelling. From this arise several questions. Firstly, is the remodelling seen in asthma a normal physiological response to bronchoconstriction *i.e.* if normal individuals were to bronchoconstrict would they remodel their airways? Alternatively are there two abnormalities in asthma, where bronchoconstriction acts on a primed airway to induce either a remodelling response where there would be none in a normal individual, or the response to the same degree of bronchoconstriction is greater in the asthmatic? Are there two abnormalities in the asthmatic; bronchoconstriction and the response to it (remodelling) or is the bronchoconstriction the only abnormality, leading to a normal physiological response?

These questions cannot be answered *in vivo* as normal individuals do not bronchoconstrict readily, even with large doses of methacholine. In order to address these questions it was apparent that bronchoconstriction would have to be modelled *in vitro* and normal and asthmatic cells exposed to forces designed to mimic bronchoconstriction.

6.1 Background

The airways are exposed to several forces during tidal breathing. There is airflow over the airway surface leading to shear stress; in addition there is cyclical expansion and contraction of the airway in response to lung expansion and contraction leading to cyclical stretch and changes in transmural pressure. The forces at work during tidal breathing, if not the responses to them, are likely to be the same in normal individuals and in patients with asthma.

In vitro stretch experiments have been performed by several groups, mostly stretching human lung fibroblasts from various stages of embryonic development on a variety of substrates using a variety of mechanical stimuli (varying frequency, magnitude and duration of stretch). These experiments have, not surprisingly, had a wide variety of outcomes, but have demonstrated that human lung fibroblasts respond to mechanical stimuli (Mascarenhas 2004, Mohammed 2007, Thomas 2006). When cocultured with bronchial epithelial cells, fibroblasts respond to intermittent mechanical stretch with increased synthesis of collagens, biglycans and proteoglycans, suggesting that mechanical stretch provides significant regulation of the growth of the lung (Tschumperlin 2006). In the transition from foetus to adult, lung water is removed and cyclical stretch of the lung occurs due to the function of respiration.

The airways are subject to mechanical stress from early in development. It has been known for many years that airway smooth muscle in the foetus is spontaneously contractile, and this has been demonstrated in animal models and in human foetuses (Schittny 2000). The onset of spontaneous contraction of the airway has been shown to be associated both with the expression of a putative asthma susceptibility gene (A disintegrin and metalloprotease (ADAM) 33) and also alpha smooth muscle actin. A further increase in expression of these two genes and proteins occurs at birth, when cyclical stretch of the airway occurs (Haitchi 2009). It has been shown that the EGFR receptor is required for normal lung development; EGFR is located in embryonic lung primordia in the epithelial and mesenchymal compartments, and EGF enhances lung maturation in rabbits and stimulates lung branching in foetal mice (Miettinen 1997). It has been demonstrated *in vitro* that compressive forces applied to epithelial cells result in EGFR ligand shedding and EGFR phosphorylation (Tschumperlin 2004); could the remodelling effects seen from cellular compression *in vitro*, mediated by EGFR, be recapitulating ontogeny. In these circumstances might it

be expected that the response to such compression would be similar in normal and asthmatic individuals?

Various *in vitro* models have been developed and used to address whether the bronchial epithelium may respond to compressive stress as a result of bronchoconstriction. Initially Ressler and colleagues examined rat tracheal epithelial cells and compressed them using an apical increase in air pressure to generate a transmembrane pressure (Ressler 2000). This work was repeated on human bronchial epithelial cells and demonstrated a release of endothelin 1 (ET-1) and ET-2 as well as TGF β 2 in response to apical stress. Only short periods of stress (minimum of 1 hour) were sufficient to commit the cells to induce signalling (Tschumperlin 2003).

This work was extended using an elegant coculture method where epithelial cells above an incompressible but porous membrane were cultured with fibroblasts below the membrane, and a transmembrane pressure applied only to the epithelial cells (shown in Figure 6-1). This demonstrated that following epithelial compression fibroblasts increased production of collagen I, III and IV in a time and pressure dependent manner (Swartz 2001).

Swartz working with others (Choe 2003) also developed a different model of epithelial compressive stress, where lateral compressive strain (rather than apical pneumatic force) is applied to co cultures of bronchial fibroblasts and epithelial cells as shown in Figure 6-2 (Choe 2003). These models have demonstrated similar findings of increased collagen I, III and IV production from fibroblasts which appeared to be orchestrated by the epithelium (Choe 2006).

Figure 6-1 Schematic diagram of coculture model of epithelial constriction leading to fibroblast response. P_o indicates pressure above atmospheric. From Swartz *et al* Proc Natl Acad Sci 2001.

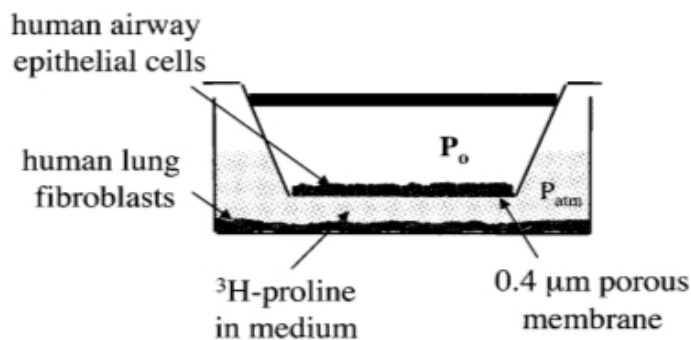
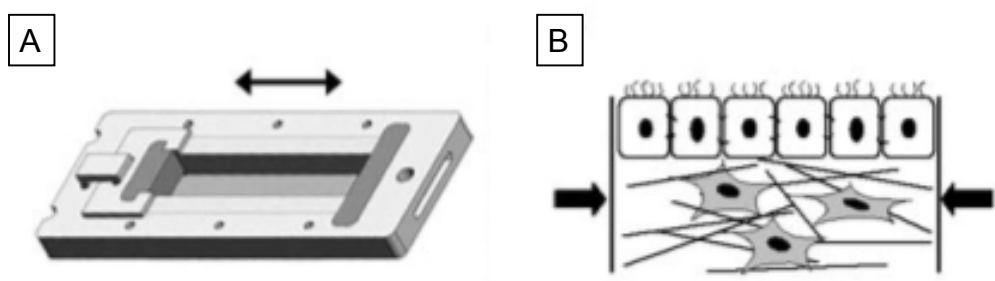


Figure 6-2 *In vitro* model of bronchoconstriction developed by Choe and colleagues. (A) Individual wells with a moveable inner wall were developed to induce lateral compressive stress to a three dimensional model of human airway tissue shown in panel B. Adapted from Choe *et al* AJRCMB 2006.



In addition to modulating remodelling, epithelial mechanical stress has been shown to alter the barrier function of the epithelium, resulting in a disruption of tight junctions and an increase in the transduction of lentivirus across the epithelial surface (Tomei 2008), although these findings are not universal, with others showing no change in FITC dextran transfer across a mechanically stressed epithelial surface (Park 2010). Apical stress on epithelial cells also appears to induce epithelial metaplasia with an increase in mucus production, even when apical stress is applied for as little as 10 minutes per day (Park 2009).

These data suggest that the epithelium is receptive to mechanical (especially compressive) stress and mediates ECM deposition following such stress, and may also undergo changes in its tight junctions and barrier function.

None of these studies however have approached the question of whether these responses are similar or different in the asthmatic population compared to non asthmatics. All the studies mentioned above have used normal human bronchial epithelial cells obtained from Lonza Clonetics® (Lonza Group Ltd, Basel, Switzerland); in this part of the study the response of asthmatic and normal HBEC's to compressive stress mimicking bronchoconstriction will be assessed.

6.2 Aims

To establish an *in vitro* model of bronchoconstriction and use it to test the hypothesis that bronchial epithelial cells from asthmatics respond the same to forces mimicking bronchoconstriction as epithelial cells from normal volunteers.

6.3 Methods and results

6.3.1 *In vitro* model of bronchoconstriction

In order to test the above hypothesis, it was necessary to construct an *in vitro* model of airway bronchoconstriction as one did not exist in the laboratory. The currently reported models of bronchoconstriction are detailed above, and the model chosen for use was the apical pressure model originally described by Ressler *et al* (Ressler 2000). This model was chosen as it is relatively easy to construct, especially when comparing it to the three dimensional collagen cocultures described by Choe *et al* (Choe 2003) and has proven ability to investigate release of profibrotic cytokines from normal primary human bronchial epithelial cells (Tschumperlin 2003, Park 2009, Park 2010).

6.3.2 Establishment of the model system

The model system was established, calibrated, tested and optimised as described in Chapter 2. Briefly a cell compression system was established similar to that initially described by Ressler *et al* (Ressler 2000) using 5% CO₂ in air from a compressed gas cylinder, a BOC supplied cylinder regulator and then a specialist low pressure regulator in series to produce pressure variability from 0 to 45 cm water pressure. This system was then used to increase the pressure over air liquid interface cultured primary human bronchial epithelial cells as required. Several problems were encountered and overcome whilst setting up and optimising the system as described in Chapter 2.

6.3.3 Volunteer recruitment

Asthmatic subjects were recruited using the criteria in Chapter 2, however anti-inflammatory medication including inhaled and oral corticosteroids were not an exclusion criterion for this element of the study. Normal individuals were recruited in a similar manner, but had no diagnosis of asthma or other respiratory disease, and did not show a bronchoconstrictive response when challenged with methacholine up to a concentration of 16mg/ml and in addition had no reversibility to salbutamol. A full list of volunteers for this element of the study is shown in Appendix 6.

6.3.4 Bronchoscopy and cell culture

Bronchoscopy was performed as described in Chapter 2. Epithelial cells were harvested by epithelial brushings and cultured to air liquid interface as also described in Chapter 2. Briefly, bronchial epithelial cells were obtained by brushing the surface of the airway with a cytology brush, the cells were then recovered into buffered saline, centrifuged and resuspended in cell culture medium. Cells were then grown to passage 2 in submerged culture, before plating onto semipermeable culture inserts (Transwells), removing the apical media, and growing them at an air liquid interface for 21 days.

6.3.5 Experimental design

In order to examine the release of remodelling mediators following a bronchoconstrictive event modelled *in vitro*, cells from 9 normal subjects and 9 asthmatic subjects were subjected to real compression for 1 hour at 30 cm of water pressure or sham compression control as described above. Each compression was carried out on 2 transwells from each donor, mean results from the two transwells were used for further analysis. The presence of TGF β 2 and RELM β were measured in the basolateral medium of the cells 24 hours after the end of compression in order to ascertain their release of pro

remodelling substances in response to compression mimicking bronchoconstriction. In a proportion of the cells (normal n=4, asthma n=4), in addition the cells were lysed into Trizol reagent at 4 hours, 8 and 24 hours after the end of compression to assess changes in gene expression for TGF β and other genes at these time points.

6.3.6 Results

TGF β 2

Total TGF β 2 was measured in the basolateral medium of cells twenty four hours after they were subjected to compression for 1 hour at 30 cm of water pressure, or to a sham compression for the same period of time.

Total TGF β 2 was increased in the asthmatic samples, but not in the medium from cells of normal subjects; Twenty four hours after sham compression the concentration of total TGF β 2 in the basolateral medium was similar in cells from normal (median (IQR) 203.4 (143.8 - 271.7) pg/ml) and asthmatic (245.5 (162.3 - 276.6) pg/ml) subjects (p=0.67). Following compression there was a significant increase in the asthmatic cell culture medium to median (IQR) of 299.4 (205.4 - 365.0) pg/ml (p=0.02) whilst there was no increase in the normal cells, median (IQR) 254.0 (137.7 - 281.7) pg/ml (p=0.68) as shown in Table 6-1 and Figure 6-3. If the difference between the sham and real compression is considered, there was a median (IQR) difference of 65.7 (24.5 - 110.7) pg/ml in the asthmatic group and 11.6 (-35.6 - 48.7) in the normal group (p=0.04 comparing between the asthmatic and normal groups) as shown in Table 6-2 and Figure 6-4.

In order to assess the concentration of active TGF β 2 in the medium from compressed cells the ELISA was repeated without acid activating the samples; there were no differences between sham or real compression or between asthmatics or normals (Table 6-3).

Table 6-1 Total TGF β (pg/ml) in basolateral medium of human bronchial epithelial cells from asthmatics and normal volunteers 24 hours after 1 hour of sham or real compression at 30cm water pressure.

	Total TGF β 2 (pg/ml)
Asthma (n=9)	
Sham compression	245.5 (162.3 - 276.6)
Real compression	299.4 (205.4 - 365.0)
p value	0.02
Normal (n=9)	
Sham compression	203.4 (143.8 - 271.7)
Real compression	254.0 (137.7 - 281.7)
p value	0.68

Values are median with IQR in parentheses. P values calculated by Wilcoxon signed rank test.

Figure 6-3 Total TGF β 2 concentration in basolateral medium of human bronchial epithelial cells from normal and asthmatic volunteers 24 hours after sham or real compression for 1 hour at 30cm water pressure.

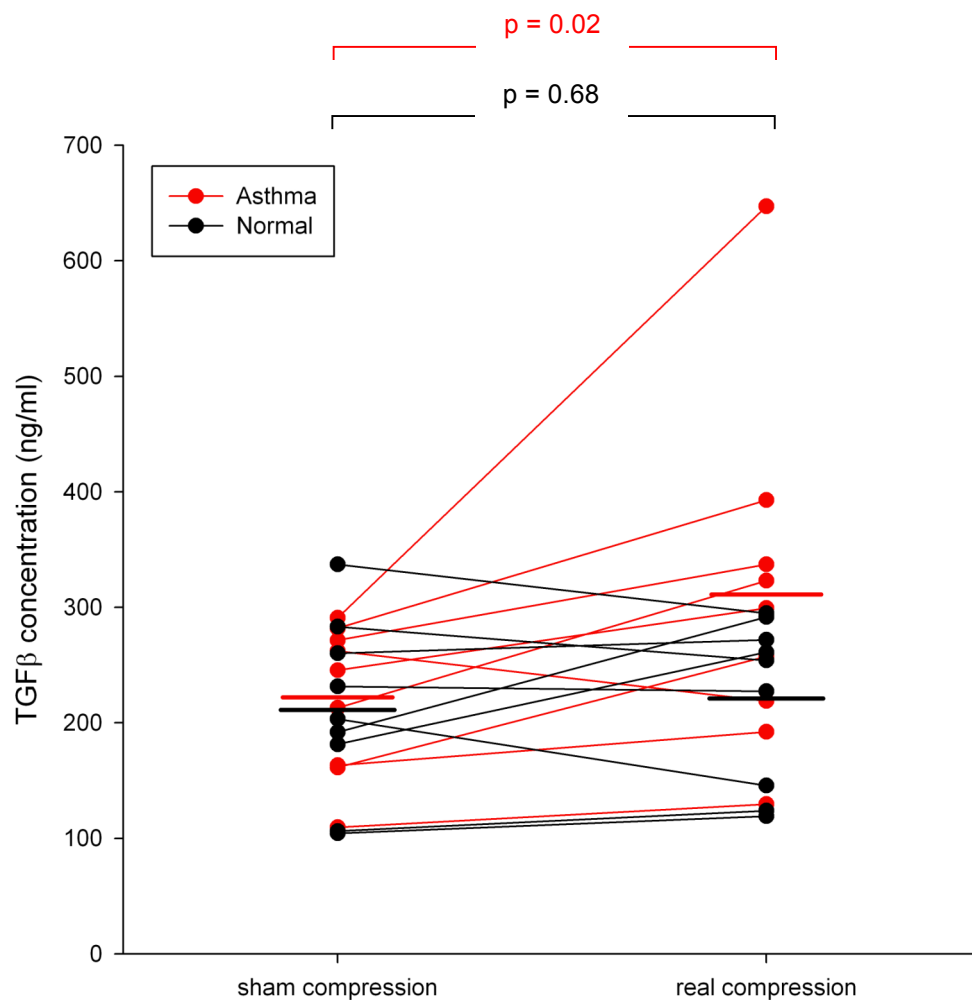


Table 6-2 Difference in total TGF β (pg/ml) in basolateral medium between cells compressed at 30 cm of water pressure for 1 hour, and those subject to sham compression.

	Δ total TGF β 2 (pg/ml)
Asthma	65.7 (24.5 - 110.7)
Normal	11.6 (-35.6 - 48.7)
p value	0.04

Values are median with IQR in parentheses. p values calculated using Mann-Whitney test

Figure 6-4 Change in total TGF β 2 concentration in basolateral medium of human bronchial epithelial cells from normal and asthmatic volunteers 24 hours after sham or real compression for 1 hour at 30cm water pressure.

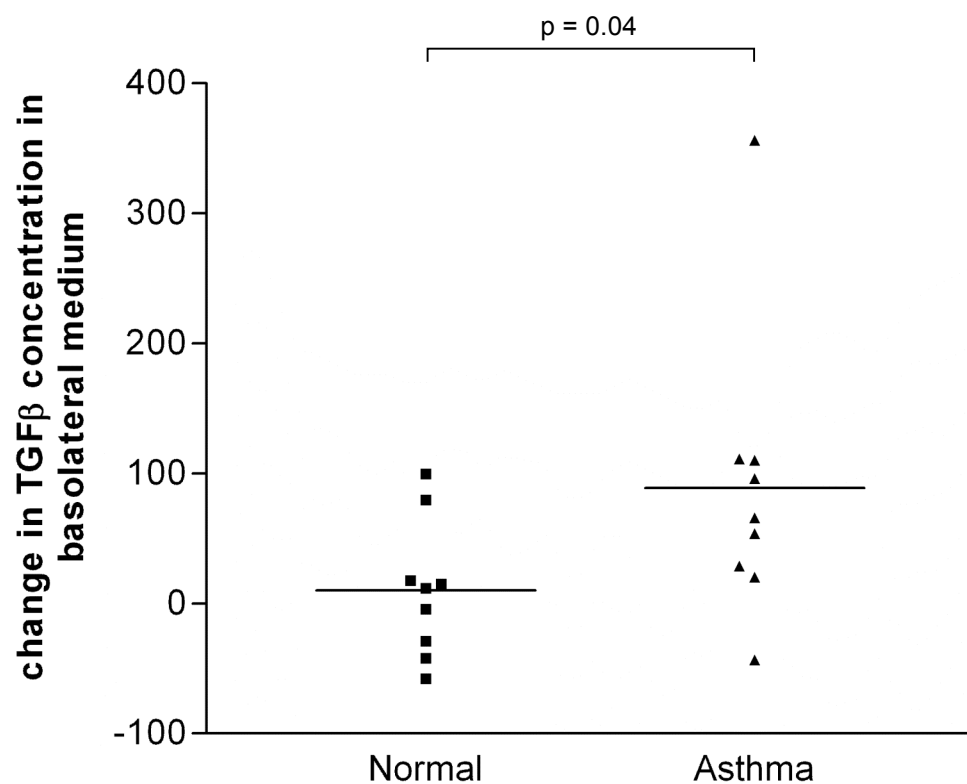


Table 6-3 Active TGF β (pg/ml) in basolateral medium of human bronchial epithelial cells from asthmatics and normal volunteers 24 hours after 1 hour of sham or real compression at 30cm water pressure.

	Active TGF β 2 (pg/ml)
Asthma (n=9)	
Sham compression	38.9 (30.1 - 53.1)
Real compression	32.3 (6.8 - 47.7)
p value	0.47
Normal (n=9)	
Sham compression	34.1 (23.7 - 63.2)
Real compression	24.6 (9.3 - 39.6)
p value	0.29
Values are median with IQR in parentheses. P values calculated by Wilcoxon signed rank test.	

RELM β was also assayed in the basolateral medium, almost all samples were below the level of detection.

qPCR results

There was no upregulation of the TGF β 2 or RELM β genes as measured by qPCR analysis at 4, 8 or 24 hours following real or sham compression (Table 6-4 and Table 6-5)

Table 6-4 Relative TGF β 2 gene expression in human bronchial epithelial cells from asthmatics and normal volunteers after sham or real 1 hour compression at 30cm water pressure.

	TGFβ2 ΔΔCT			
	4 hr	8 hr	24 hr	p value
Asthma (n=4)				
Not compressed	1.0 (1.0 - 1.0)	1.0 (0.8 - 2.1)	0.9 (0.7 - 4.7)	0.72
Compressed	0.4 (0.4 - 1.5)	0.6 (0.4 - 1.1)	0.8 (0.3 - 2.5)	0.72
Normal (n=4)				
Not compressed	1.0 (1.0 - 1.0)	1.0 (0.7 - 1.1)	1.8 (0.6 - 3.1)	0.80
Compressed	0.7 (0.6 - 1.1)	0.6 (0.4 -0.9)	0.8 (0.5 -1.4)	0.47

p values calculated with Friedman's test. values are median and IQR in parentheses. Values calculated by $\Delta\Delta$ CT method using the 4 hour sham compressed sample for each group as the reference.

Table 6-5 Relative RELM β gene expression in human bronchial epithelial cells from asthmatics and normal volunteers after sham or real 1 hour compression at 30cm water pressure.

	RELMβ ΔΔCT			
	4 hr	8 hr	24 hr	p value
Asthma (n=4)				
Not compressed	1.0 (1.0 - 1.0)	0.4 (0.3 - 8.4)	1.0 (0.5 - 4.9)	0.72
Compressed	0.4 (0.4 - 4.4)	0.6 (0.4 - 1.1)	1.5 (0.4 - 4.9)	0.72
Normal (n=4)				
Not compressed	1.0 (1.0 - 1.0)	0.9 (0.4 - 3.2)	1.4 (0.9 - 7.9)	0.17
Compressed	0.8 (0.2 - 1.8)	0.9 (0.5 - 1.8)	0.7 (0.5 - 2.1)	0.37

p values calculated with Friedman's test. values are median and IQR in parentheses. Values calculated by $\Delta\Delta$ CT method using the 4 hour sham compressed sample for each group as the reference.

6.4 Discussion

This series of experiments have demonstrated that cells from asthmatic patients respond differently from normals to a compressive force *in vitro* that mimics bronchoconstriction. This has not been previously demonstrated and suggests that there are at least two abnormalities in the asthmatic airway; the initial bronchoconstriction, and the epithelial response to it.

6.4.1 Absent response from normal cells to compression

Above it was shown that the airway responds to mechanical stress during development, and the stresses and development of the lungs appears similar (on a macro scale at least) in asthmatic and normal lungs. If the normal and asthmatic lung are both capable of responding to mechanical stress, why then did the normal cells not respond to mechanical stress in this instance? It is possible that it was the wrong sort of stress; the stresses that the lungs are exposed to in development, and indeed normal breathing are likely to be stretch forces and shear stress over the surface of the airway epithelium, not compressive force as would be applied during bronchoconstriction. It may be that normals respond to stretch and shear stress (as has been shown) but that they don't respond (at least to the same degree) to compressive stress. This would be disputed by the work of Tschumperlin, Swartz *et al* who using normal human bronchial epithelial cells have demonstrated a response from the epithelium to mechanical stress similar to that generated here. Why might the normals in our study not respond to mechanical stress?

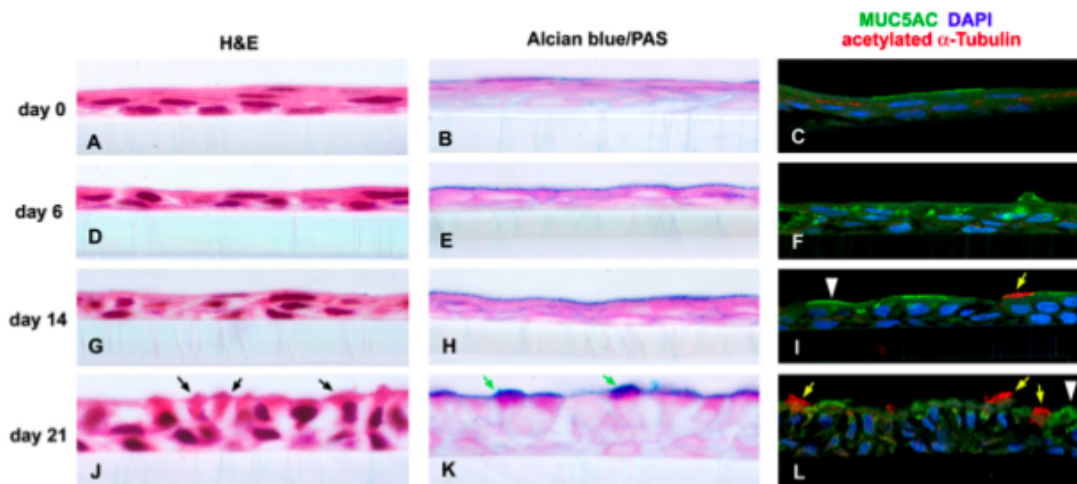
It may be that the mechanical stress induced experimentally in this study is different to that induced by Ressler *et al*, though this is unlikely as great care was taken establishing the apparatus and calibration has shown that the expected pressures are generated at the cell surface. It may also be that the

cells used in our study were in some way different to those used in the other studies. Notably some of the normal individuals in our study did respond to mechanical stress, and whilst we used a relatively large number of donors, previous studies have used 2 or 3 donors at the most.

There may be other experimental differences between the two approaches; all previous *in vitro* studies used normal HBEC's from Lonza Clonetics® (Lonza Group Ltd, Basel, Switzerland), which are all frozen for transport. This may affect the physiological responses of the cells; all of the cells used in this project were cultured immediately following removal at bronchoscopy. Some preliminary data in our laboratory suggests that frozen then thawed cells may respond differently to non frozen cells (Cakebread, personal communication). This could be established relatively easily and quickly by freeze thawing some cells from a specific donor, then conducting the same experiments as above.

In addition to using previously frozen cells, Tschumperlin *et al* use their cells in ALI culture cells after they have been passaged 4 times, whereas ours are used at passage 2 which could again alter the responses. This would also be relatively easy to investigate. The most likely cause however for the differences seen is the stage of development at which the ALI cultures have been used in previous studies compared to the current work. Previous studies have been performed on ALI cultures after only between 10-14 days at air liquid interface (Tschumperlin 2003). At this point the cells are still differentiating into a true epithelial phenotype, whereas our cells were maintained at air liquid interface for at least 21 days prior to use. Immunofluorescence studies of ALI cultures shows that at 14 days after establishment at ALI the cells remain in a stratified squamous epithelial layer, with little evidence of ciliation (Figure 6-5) whereas by day 21 the cells are arranged in a pseudostratified pattern and the cells have ciliated (Ross 2007). Using more sensitive techniques (electron microscopy) it has been shown that ciliogenesis starts around 7 days after taking cells to air liquid interface, but is not complete until 31 days (de Jong 1994).

Figure 6-5 Mucociliary differentiation of primary human bronchial epithelial cells during ALI culture. The incomplete differentiation of cells up to day 14 is clearly demonstrated, with fully differentiated cells being seen at 21 days. Cells were collected at day 0, 6, 14 and 21 following establishment of air liquid interface, fixed and sectioned. Sections were stained with H&E stain (left column), Alcian blue and periodic acid-Schiff (PAS) reagent (middle column) or immunostained to label acetylated α -tubulin (red), MUC5AC (green) and nuclei (blue) (right column). Ciliated cells are indicated with black arrows and cells with high levels of PAS staining with green arrows. Acetylated α -tubulin was observed on the surface of ciliated cells (yellow arrows) and high levels of mucin staining were seen on the surface of secretory cells (white arrowheads). Adapted from Ross *et al* Am J Respir Cell Mol Biol 2007.



This difference in maturity may be the main cause of the differences seen between our study and previously published work; the cells used by Tschumperlin *et al* are likely to be less well differentiated and hence more plastic and may therefore respond differently to any physical stressor. Cellular stress at the earlier time point may have more effect than compression later, this may have relevance if the epithelium is damaged due to viral infection or other environmental challenge. If bronchoconstriction were to occur during this time it could be postulated that it may have an even greater impact on the remodelling process. In order to investigate this further, cells from healthy and asthmatic donors could be compressed at various points after establishing them at the air liquid interface. In essence what has been shown by the previous work is that normal HBEC's respond to compressive stress during differentiation, not that they respond to compressive stress once fully differentiated.

6.4.2 Further work

One of the disadvantages of using ALI cultures is that there is very little basolateral medium (300µl per well) which restricts analysis by standard ELISA techniques. Because of this only three ELISA's have been performed on the medium; total and activated TGFβ2 and RELMβ. In order to assess other cytokines that might be of interest, analysis of the remaining culture medium is planned using a bead based multiple cytokine assay (Luminex, Austin, USA) (Lash 2006).

This data suggests that the bronchoconstriction in asthma acts on a primed epithelium to induce remodelling changes that would be absent or reduced in normal individuals undergoing the same degree of bronchoconstriction. The implications for this are discussed in the final chapter.

Chapter 7 Conclusions

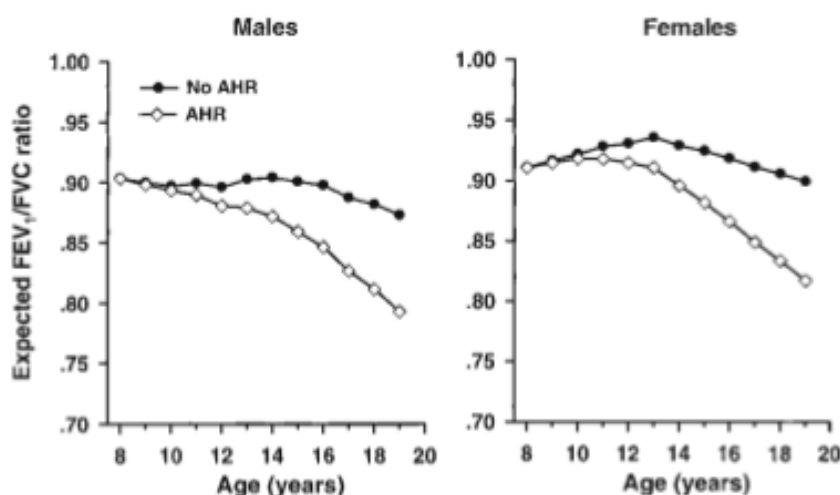
The aim of this project was to examine the hypothesis that *'repeated bronchoconstriction in the absence of inflammation will induce a different airway remodelling response to repeated bronchoconstriction with the addition of airway inflammation'*. This hypothesis has been disproved with the demonstration that methacholine induced bronchoconstriction induces similar remodelling responses in the airway as allergen induced bronchoconstriction.

In addition we demonstrated that the response to bronchoconstriction is not the same in asthmatics and healthy individuals, with a greater response *in vitro* to compressive stress in asthmatic human bronchial epithelial cells.

7.1 Implications for early treatment of asthma and lung development

This study has implications when considering airway remodelling in childhood and in lung development. Airways hyperreactivity and/or wheeze is associated with a reduced rate of growth in airway calibre as measured by FEV₁ and FEV₁:FVC ratio. This results in 3% lower FEV₁ than if subjects have no recent wheeze, as found in a longitudinal study of Australian children and shown in Figure 7-1 (Xuan 2000). Our work suggests that the presence of bronchoconstriction / wheeze induces the deposition of connective tissue, which could then limit the growth of the airways as found. Thickening of the airway has also been found in the absence of eosinophilic inflammation in other studies in children (Cokuğras 2001). Notably these studies use wheeze as a definition of asthma which as a marker of bronchoconstriction may now be said to contribute to airway thickening.

Figure 7-1 The growth pattern of expected FEV₁/FVC ratio in boys and girls with and without airways hyperreactivity. From Xuan *et al* Am J Resp Crit Care Med 2000.



Our findings that bronchoconstriction may induce airway remodelling help to explain the lack of long term beneficial effects of regular inhaled corticosteroid treatment in either preschool or school age children (Guilbert 2006, Childhood Asthma Management Program Research Group 2000). Whilst these studies focussed on treating the inflammatory element of asthma, they did not address the bronchoconstriction element of the disease. This may have which left the epithelium responsive to compressive stress from bronchoconstriction as we have demonstrated, leading to collagen deposition within the airway and an effect on lung function. We did not investigate whether corticosteroids have an impact on bronchoconstriction induced airway remodelling, it would be interesting to examine the remodelling effects of bronchoconstriction on patients being treated with inhaled or oral steroid therapy.

The treatment of pre-school and school age children with corticosteroids may also have failed to induce any long term beneficial changes if the underlying

pattern of lung development had been fixed earlier than the point of intervention. It is well recognised that the lungs are exposed to, respond to and are dependent on mechanical stress in order to develop correctly (Tschumperlin 2006, Haitchi 2009). Our data may offer a window into the development of the lungs in asthma; we have demonstrated that asthmatic and normal human bronchial epithelial cells in our study responded differently to mechanical stress. It is therefore possible that the mechanical stress encountered during development triggers different responses in asthmatic and normal cells; this in turn could result in developmental differences which are revealed during early life as alterations in lung function. During development, any differences in response to mechanical stress are likely to be genetically mediated, and many of the asthma susceptibility genes identified are expressed during lung development and expressed in epithelial and mesenchymal tissues (Holloway 2010b, Carpe 2010). This hypothesis may of course may not be true; the cells that were compressed *ex vivo* in this study were removed from adult asthmatics and then grown to a putative adult phenotype *in vitro* prior to compression. The cells would almost certainly have a different phenotype *in utero*, perhaps more akin to the less developed cells compressed by other groups *in vitro* (Park 2009). It would be possible, though difficult, to obtain human foetal bronchial epithelial cells and examine their response to mechanical stress, though it would not be possible to determine their asthma phenotype to assess difference in response between asthmatics and normals, at least until our understanding of the genetics of asthma becomes more complete.

7.2 The role of bronchodilators in airway remodelling

Our data also allows reinterpretation of asthma remodelling studies performed and interpreted without knowledge of our data. Kelly *et al* recently showed that remodelling in the airway following allergen challenge was decreased by combination long acting β -agonist (LABA) and inhaled steroid therapy, whilst inhaled steroid alone did not reduce the remodelling (Kelly 2010). This study

was interpreted as the LABA acting synergistically with the inhaled steroid to reduce inflammation and the remodelling, even though there was no significant reduction in the eosinophil counts in the LABA plus steroid group compared to the steroids alone. Although it appears in cell culture that LABA's have a direct effect on cells, decreasing their inflammatory response to airway triggers (Skevaki 2009) it is now possible to speculate that at least some of the reduction in remodelling in the Kelly study was due to the reduction in bronchoconstriction caused by the LABA. These findings do not only appear with bronchodilation with beta agonists; Gosens *et al*, examining the remodelling of smooth muscle in guinea pigs following OVA challenge, showed that remodelling was reduced following anticholinergic therapy with tiotropium (Gosens 2005). This study was interpreted that the tiotropium given to the OVA challenged animals was blocking ACh mediated remodelling, whereas it may have been that the remodelling was reduced subsequent to the reduction in bronchoconstriction. If two bronchodilators acting by different mechanisms both prevent bronchoconstriction (as is demonstrated in both the Gosens *et al* and Kelly *et al* studies) it is possible that they are reducing remodelling by different mechanisms, but it would be a simpler, and now biologically plausible explanation, that they are reducing remodelling by their shared effect in preventing bronchoconstriction.

7.3 The EMTU and airway remodelling

An relatively recent asthma paradigm is that of the epithelial mesenchymal trophic unit (EMTU) acting as a central component of the airway through which both genetics and environment interact to influence the clinical and pathological expression of asthma (Holgate 2009). This paradigm suggests that the epithelium is a key structural and signalling cell which promotes recruitment of cells into the airway, and also signals to mesenchymal fibroblasts to induce myofibroblast transformation, leading to a wound repair response (Zhang 1999). Our study supports the EMTU paradigm, with epithelial TGF- β being generated

in response to mechanical stress from bronchoconstriction. The epithelium's role in the production of airway collagen is further reinforced by the fact that sub-basement membrane collagen (close to the epithelium) was increased in both the allergen and methacholine groups, but there was no increase in submucosal collagen density (more distant from the epithelium). *In vitro* models have shown greater collagen deposition just beneath the epithelium in response to mechanical stress (Choe 2006) however these models do not contain other potential effectors of airway remodelling such as eosinophils and airway smooth muscle. It may be that fibroblasts at different levels of the airway are driven or controlled by different tissues; the fibroblasts just deep to the epithelium may be subject primarily to epithelial control, whilst those near the smooth muscle may be much more dependent on smooth muscle signalling (Kaur 2006). This then raises the possibility that remodelling by these different populations of fibroblasts is stimulated and regulated independently; remodelling may not be a uniform process, rather it may be differentially expressed in different parts of the airway depending on the exact stimulation provided and the underlying genetic susceptibility of the individual.

Potential stimuli to airway remodelling include amongst others, mechanical stress, inflammation, ACh release and smooth muscle mediator release; they are all likely to act on a genetically primed airway. Genome wide association studies have shown linkage of the asthma phenotype to several genetic loci and specific genes which appear to be involved in both smooth muscle (Phosphodiesterase E3 duncce homolog, *Drosophila* (PDE4D)) and epithelial (ORMDL3 and Gasdermin B (GSDMB)) elements of the airway (Holloway 2010a). It may be that the asthma phenotype is dependent on the interaction of a variety of genes within a genetic network, several of which have to be abnormal in order to result in the asthma phenotype. It is possible that one of many different genes has to be abnormal in order to result in AHR, whilst another gene from an 'epithelial bank' of genes has to be abnormal in order to result in the airway remodelling and ongoing EMTU activation. This might

explain why there is not stronger linkage with easily identifiable single gene abnormalities in the genetic studies. Our data support this 'two hit' hypotheses as both the smooth muscle (in causing AHR to methacholine) and the epithelium (in orchestrating a response to bronchoconstriction) have been found to be independently abnormal in our study.

7.4 Measurement of airway hyperreactivity in asthma

The findings of our study have implications for the use of methacholine and other bronchoconstrictive challenges in the investigation of asthma, and specifically in any studies of airway remodelling. It will now not be possible to examine changes both in airway hyperreactivity and remodelling in the same study group; additional control groups will be required if assessments of AHR are to be made. Previous studies which did assess AHR and remodelling, such as that performed by Kariyawasam *et al*, should be reviewed in light of our findings (Kariyawasam 2007). In their study they exposed volunteers to allergen challenge and then methacholine challenge prior to bronchoscopy, and attributed the remodelling changes to the allergen challenge, discounting the possibility that bronchoconstriction *per se* could have induced the changes seen.

Recent *in vitro* work has shown that fibrosis increases the stiffness of lung tissue much more than previously expected. In addition, normally the proliferation of lung fibroblasts is suppressed, but when the lungs become stiffer due to interstitial fibrosis both fibroblast proliferation and procollagen I synthesis is increased, inducing an adverse feedback cycle (Liu 2010). Although this data was obtained from a bleomycin induced mouse model, it may be that a similar response is seen in human asthmatics where initial bronchoconstriction might lead to remodelling, which then becomes more likely due to stiffer lung tissue. If an initial allergen challenge caused some increase in lung stiffness by inducing remodelling, then perhaps any further bronchoconstriction might have a greater

remodelling effect than if the lung is not primed. This makes the avoidance of AHR measurements following experimental allergen challenge vital in the future, it may also offer an explanation as to why the repeated challenges used in this study gave a positive response.

7.5 Should these findings change our management of clinical asthma patients?

The results from this small preliminary study are interesting, but alone should not fundamentally alter the management of our clinical patients, however it is worth considering whether the findings that bronchoconstriction induces remodelling might at some point either change the emphasis of treatment, or if the understanding of the underlying process may improve patient outcomes. Current asthma treatment guidelines such as those issued by the British Thoracic Society appropriately concentrate on anti inflammatory medication as the mainstay of treatment in order to achieve outcomes of better asthma quality of life, decreased exacerbation rates and reduced asthma deaths (BTS/SIGN 2008). LABA's produce bronchodilation and improve asthma symptoms, and those effects are maintained with regular use over time, and currently LABA's are recommended to be added to therapy when inhaled low dose corticosteroids are insufficient to control symptoms (BTS/SIGN 2008). In light of our findings that bronchoconstriction may induce long term changes within the airway, it might be worth speculating, that with additional evidence, LABA's might be considered with an inhaled corticosteroid at Step 2 of treatment. This is likely to be controversial as there are ongoing safety concerns regarding the use of LABA's. Although these drugs improve symptoms and reduce the risk of asthma exacerbations, they may worsen asthma by negative feedback of the β -adrenergic system by an adaptive response to stimulation of receptors, though they are safer with concurrent inhaled corticosteroids (reviewed in Chung 2009). Recent meta analysis of the clinical trials examining LABA use have concluded that there is an increased risk of hospitalisations due to asthma, life-threatening

asthma exacerbations, and asthma-related deaths from lone (without corticosteroids) use of LABA's (Salpeter 2006). These findings are not without controversy themselves, as the majority of the patients in the meta analysis were from a single study, the Salmeterol Multicentre Asthma Research Trial (SMART) (Nelson 2006). All but two asthma deaths in the meta analysis were from the SMART trial, and the SMART trial recruited a significant proportion of its subjects from the African American community who were over represented in the asthma related deaths; non African American participants in the trial did not show an increased risk of death. There may be many reasons for this including poor underlying asthma control, less access to health care and also the high prevalence of a variation in the β adrenoceptor gene (homozygous for arginine at position 16 (Arg/Arg genotype)); people with such genetics are known to experience decline in airflow and worse asthma control when given β adrenoceptor antagonists (Israel 2000). In the majority of the UK population, this is unlikely to be an important factor; in addition LABA's are almost always prescribed with inhaled corticosteroids in this country.

In light of the findings of this study, were they to be replicated in other work, the anti inflammatory goal directed therapy in asthma might be augmented with another goal, that of preventing bronchoconstriction as a specific aim.

7.6 Resistin like molecule beta (RELM β)

Resistin like molecule beta (RELM β) has not previously been examined in human airways either in health or disease. We demonstrated that in the airway epithelium it is immuno-expressed to a greater degree in more severe asthmatics, and that its expression is increased following allergen or methacholine challenges. Animal data has shown that RELM β is necessary and sufficient to induce airway remodelling and is chemottractive to human fibroblasts *in vitro* (Mishra 2007). Its increased expression following allergen and methacholine challenges, in conjunction with increased immunoexpression

of TGF β raises the possibility that is an important remodelling mediator not only in animals, but also in humans. It is possible that RELM β acts in concert with TGF β , with RELM β acting as a fibroblast chemottractant, recruiting fibroblasts to the subepithelial space, with TGF β inducing their transformation into myofibroblasts with subsequent collagen production. It has been shown that RELM β mRNA is upregulated in homogenised mouse lungs following stimulation with IL4 or IL13. We could not detect RELM β mRNA at rest or following stimulation with either or both of these in human fibroblasts, which may suggest either a different role for RELM β in the animal model, or that another cell type in humans is responsive to stimulation; our data would suggest that this is the epithelium. It would be interesting to examine the effects of IL4 and IL13 on the epithelial expression of RELM β *in vitro*.

7.7 Limitations of this study

This study is limited in several important ways which should be considered when interpreting its results.

The numbers in the study were small, 12 in each study group, and whilst statistically and clinically significant results were obtained, we are limited by the small numbers in drawing conclusions on some measures, especially for any potential differences between the allergen and methacholine groups, but also potentially any small remodelling contribution from a direct effect of methacholine which may have been hidden by small numbers.

Multiple measures of airway remodelling were made and it is possible that the statistically significant results arose purely by chance as a result of multiple analysis. This is considered unlikely as the results follow a biologically plausible pattern, and many of the p values are highly significant, but it should be considered. Ideally, correction for multiple analysis would have been performed, however with the small sample size, this would have reduced the risk of a type I

error at the expense of a greatly increased risk of a type II error. In order to address the possibility that repeated analysis lead to the statistically significant results seen, the ideal would be to repeat the study in a different population using larger numbers.

The population from which our volunteers was drawn was not truly representative of the general asthmatic population. Just under 50% of asthmatics are only receiving short acting β -agonists as therapy (Liard 2000), whereas all of the study population were on such treatment. In addition our study had a preponderance of female volunteers and the age of the participants was generally quite low, reflecting the large proportion of student volunteers. These factors mean that the study population does not reflect the general adult population with asthma, and the implications of this should be considered when interpreting the study. It is possible that the rapid remodelling responses that were seen are only prevalent in the relatively young age group which was sampled here, older asthmatics may not have such responsive airways, and hence the implication that airway narrowing may be an appropriate target for therapy may only be true in a young population. Patients who have undergone repeated bronchoconstriction for many years may develop a more fixed airway pathology which is less responsive to acute stimulation, this was not addressed here, but could be by specifically selecting, or stratifying volunteers by age. The more severe asthmatics who were excluded from the study may respond differently to repeated bronchoconstriction, and this would be interesting to investigate, though would be limited by safety of inducing bronchoconstriction in less well individuals.

The study also only examined the remodelling responses a short time after the bronchoconstrictive stimuli and at a single time point. This study does not address at all the natural history of the regression of remodelling or its maximal extent, one of which must occur if there is not to be a unrelenting deposition of collagen within the airway. As such the study is limited in its approach. Ideally

the same volunteers would have been bronchoscoped again at intervals to examine the regression or chronicity of the remodelling process, however this would have ethical and practical problems, such as where to biopsy in the airway to ensure biopsies were not inducing a repair response.

7.8 Future work

This study demonstrates that mechanical stress in the airway results in airway remodelling in asthma. It also suggests that remodelling is mediated via epithelial mesenchymal interaction, however it did not investigate the possibility that remodelling is mediated, at least in part, by eosinophils within the airway.

There are two ways in which the role of eosinophils might be investigated in the remodelling process, *in vivo* and *in vitro*. Considering the *in vivo* first, it would be possible to deplete circulating and to an extent, tissue eosinophils by pre-treating volunteers with mepolizumab (Flood-Page 2003, 2007), once the eosinophils were depleted, their contribution to bronchoconstriction induced remodelling could be assessed. Alternatively, it would be possible to expose volunteers to the same allergen challenge protocol as performed in this study, except with the complete abrogation of bronchoconstriction using a combination of short and long acting β -agonists to prevent the early and late asthmatic responses. By doing this, the relative contribution of the eosinophil influx into the tissues following allergen challenge could be assessed.

It is possible that eosinophils are responsive to mechanical stress and that the mechanical stress induced by bronchoconstriction in this study did not act at the epithelium, but rather via eosinophils already present in the airway; this could be investigated *in vitro*. Eosinophils could be isolated from the blood of asthmatics and normal volunteers, then exposed to apical pneumatic stress in the same manner as the bronchial epithelial cells in this study, and their activation responses measured. This might have several practical problems, as

the plating of the cells onto plastic may alter their responses; it may be that they would have to be seeded into a collagen matrix in order to mimic the *in vivo* architecture more closely. The hypothesis that eosinophils may be directly activated by mechanical stress and therefore induce remodelling responses via this pathway does however need to be investigated. Once the responses of eosinophils alone to mechanical stress were established, it would then be possible to go on to perform co-culture work combining eosinophils, epithelial cells and fibroblasts.

In our study there was no significant difference between the remodelling seen in the allergen and the methacholine challenged groups. This has been discussed previously as possibly due to a threshold of bronchoconstriction beyond which there is no difference in the remodelling changes induced. In order to address this using our model it would be possible to bronchoconstrict a group for a very short time period then examine by bronchoscopy the effects on remodelling markers. For example bronchoconstriction could be induced with methacholine, then terminated using a bronchodilator after 10 minutes, using for comparison a group of volunteers that were not treated with a bronchodilator. Also the different remodelling effects of different bronchoconstrictors should be examined to establish definitively whether the constrictive agent plays a significant role. We controlled for direct action of methacholine using salbutamol then methacholine, but investigation of remodelling following histamine, mannitol or eucapnic voluntary hyperventilation (Anderson 2003) should be performed to definitively rule out any direct role of methacholine acting at receptors within the airway rather than acting via the bronchoconstriction pathway.

We demonstrated that apical mechanical stress induces responses in human bronchial epithelial cells. However it is likely that epithelial cells are also exposed to greater airway shear stress during bronchoconstriction (Cebal 2004), and it has been demonstrated that alterations in shear stress can affect

airway epithelial cells (Abraham 2006, Tarran 2005). As the apical stress model has been established, a shear stress model, using circumferential force to induce shear stress (Button 2007) could also be established relatively easily. This would allow the combination of increased shear stress and increased apical stress to be investigated simultaneously to more accurately mimic the role of mechanical stress during bronchoconstriction.

The study was only able to examine the thickness of the airway at one time point following repeated inhalation challenge. No data is available regarding the speed of development or resolution of the airway changes using this protocol. Repeated interventional measurement of airway remodelling is technically difficult, expensive and demanding of volunteers. Ideally a non interventional method of repeatedly assessing airway thickness could be used to track the development of airway remodelling and any resolution. This might be possible with endobronchial ultrasound (Shaw 2004), even though this does not require biopsies, it still requires repeated bronchoscopy with many of its inherent difficulties. Non interventional measurement of the thickness of the airways has been performed previously using helical CT scanning (Niimi 2003) and this would be worth investigating in future. This approach has a radiation burden to the volunteers that would have to be considered carefully, though restricted dosing windows and helical scanning reduce this compared to conventional imaging (Niimi 2000).

7.9 Summary

This study demonstrates that repeated high dose inhalation allergen challenge in human mild asthmatic volunteers is safe and acceptable, and that such challenge does not produce different airway remodelling to repeated inhaled methacholine challenge when matched to initial bronchoconstrictive response. In addition it has shown that primary human bronchial epithelial cells react differently to mechanical stress if they are from asthmatic patients compared to

those from healthy controls. These findings have important implications for the understanding of asthma airway remodelling pathogenesis, for the investigation of asthma and potentially for its treatment.

Chapter 8 Appendices

8.1 Study exclusion criteria

Current smoker, ex-smoker who quit <1yr prior to study and smoking history >10 pack years

Diagnosis or documented history of bronchopulmonary aspergillosis or uncontrolled infections

Any clinically significant cardiopulmonary abnormalities not related to pre-existing asthma

Past or present tuberculosis, systemic lupus or multiple sclerosis

Any clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment

History of psychiatric, medical or surgical disorders that may interfere with study

Clinical history suggestive of respiratory infection in month preceding study

Alcohol or recreational drug abuse

Diagnosis of immunodeficiency requiring treatment

Treatment with immunomodulators (inhaled corticosteroids in two months or oral corticosteroids in six months prior to study)

Ongoing allergen desensitisation therapy

Regular use of sedatives, hypnotics, tranquillisers

Positive hepatitis viral antigens or antibodies

Blood donation within 3 months of the study

Live immunisation <4 wks prior to study

Inability to understand directions for study assessment

Inability to be contacted in case of emergency

Participation in another study concurrently or within a prior 3-month period

8.2 Symptom diary card

Asthma symptom diary card

Morning diary

Please do the breathing test and fill in the diary before taking your medication.

Please write in the number that best describes how your asthma has been during the night and this morning (think about how your asthma has been since you filled in this diary last night).

DATE							
Peak expiratory flow rate Please record the best of three blows before you take any asthma medication							
How often were you woken by your asthma during the night? 0 not woken at all 1 once 2 a few times 3 several times 4 many times 5 a great many times 6 awake all night							
How bad were your asthma symptoms when you woke up this morning? 0 no symptoms 1 very mild symptoms 2 mild symptoms 3 moderate symptoms 4 quite severe symptoms 5 severe symptoms 6 very severe symptoms							

Bedtime diary

Please write in the number that best describes how your asthma has been during the day today (think about how your asthma has been since you filled in this diary this morning).

DATE							
Peak expiratory flow rate Please record the best of three blows before you take any asthma medication							
How limited were you in your activities today because of your asthma? 0 not limited at all 1 very slightly limited 2 slightly limited 3 moderately limited 4 very limited 5 extremely limited 6 totally limited							
How much shortness of breath did you experience today? 0 none 1 a very little 2 a little 3 a moderate amount 4 quite a lot 5 a great deal 6 a very great deal							
How much of the time did you wheeze today? 0 not at all 1 hardly any of the time 2 a little of the time 3 a moderate amount of time 4 a lot of the time 5 most of the time 6 all of the time							
Please record the total number of puffs/inhalations of bronchodilator () you have used in the past 24 hrs							

8.3 Main study volunteer characteristics

Group	Sex	Age	% pred FEV ₁	% pred FVC	PC ₂₀ (mg/ml)	Spt (HDM)	Spt (Hist)	Wt (Kg)	Ht (m)	BMI
Allergen	Female	26	98.68	106.94	2.33	7	6	57	1.58	22.83
Allergen	Male	23	95.42	96.67	1.59	5	3	84	1.96	21.87
Allergen	Female	22	91.69	97.99	0.45	5	3	75	1.69	26.26
Allergen	Male	23	92.79	91.33	0.87	8	6	80	1.8	24.69
Allergen	Female	22	92.74	114.39	2.73	7.5	3	78	1.74	25.76
Allergen	Female	25	85.17	95.04	1.93	5.5	3	60	1.62	22.86
Allergen	Female	23	107.02	112.57	0.81	8	3.5	58	1.56	23.83
Allergen	Female	22	110.32	110.55	0.55	4.5	3	59	1.68	20.9
Allergen	Female	21	88.56	104.07	0.07	10	3.5	71	1.72	24
Allergen	Female	21	115.38	121.25	1.84	6.5	5	67	1.68	23.74
Allergen	Male	21	110.88	109.15	5.46	7	2	85	1.83	25.38
Allergen	Female	22	93.02	99.42	0.14	8	4	57	1.56	23.42
Allergen	Female	40	98.28	108.63	0.3	3	4.5	80	1.64	29.74
Allergen	Male	23	114.74	107.82	6.66	7.5	3.5	70	1.68	24.8
Allergen	Female	21	76.76	78.5	1.32	8.5	5	52	1.55	21.64

Group	Sex	Age	% pred FEV ₁	% pred FVC	PC ₂₀ (mg/ml)	Spt (HDM)	Spt (Hist)	Wt (Kg)	Ht (m)	BMI
Allergen	Female	30	96.89	102.4	0.53	8	7.5	55	1.58	22.03
Methacholine	Male	26	81.61	94.83	0.06	11	4	100	1.88	28.29
Methacholine	Female	22	107.53	110.85	2.93	3	4	62	1.73	20.72
Methacholine	Female	22	70.57	77.63	5.28	6	3	69	1.64	25.65
Methacholine	Female	33	131.49	134.73	1.32	3	3	60	1.6	23.44
Methacholine	Female	24	115.8	116.95	5.04	3.5	2	60	1.74	19.82
Methacholine	Male	20	84.91	100.61	0.18	10.5	5	58	1.7	20.07
Methacholine	Male	56	98.07	113.7	2.35	10	4.5	65	1.67	23.31
Methacholine	Female	18	99.18	115.11	3.69	3	6.5	70	1.7	24.22
Methacholine	Female	23	87.16	104.88	0.17	8	4	54	1.62	20.58
Methacholine	Male	21	81.98	76.87	1.39	8	6	70	1.78	22.09
Methacholine	Female	19	102.53	105.19	0.68	9.5	4.5	63	1.67	22.59
Methacholine	Female	21	111.54	114.61	0.95	3	4	80	1.58	32.05
Placebo	Female	31	97.13	100	7.89	13	5	63	1.55	26.22
Placebo	Male	19	78.62	91.11	1.47	6.5	5	63	1.71	21.55
Placebo	Female	21	95.98	95.58	0.21	5	5.5	65	1.79	20.29
Placebo	Male	18	133.67	132.88	1.87	10.5	5	90	1.85	26.3
Placebo	Female	20	107.8	104.06	5.89	2.5	5	57	1.65	20.94
Placebo	Female	20	96.21	105.7	2.27	2.5	4	73	1.72	24.68
Placebo	Female	20	92.49	109.9	2.86	3.5	5.5	59	1.65	21.67

Group	Sex	Age	% pred FEV ₁	% pred FVC	PC ₂₀ (mg/ml)	Spt (HDM)	Spt (Hist)	Wt (Kg)	Ht (m)	BMI
Placebo	Female	19	103.08	118.11	1.34	8	2.5	60	1.62	22.86
Placebo	Male	20	99.56	110.04	1.07	6	2.5	65	1.78	20.52
Placebo	Male	24	106.94	112.82	1.2	10.5	5	82	1.88	23.2
Placebo	Female	20	73.96	90.39	0.12	10	2	70	1.63	26.35
Placebo	Female	20	94.08	94.81	2.14	10.5	5	50	1.62	19.05
Salbutamol	Female	30	96.89	102.4	0.53	8	7.5	55	1.58	22.03
Salbutamol	Female	19	102.53	105.19	0.68	9.5	4.5	63	1.67	22.59
Salbutamol	Female	20	107.8	104.06	5.89	2.5	5	57	1.65	20.94
Salbutamol	Female	22	92.74	114.39	2.73	7.5	3	78	1.74	25.76
Salbutamol	Male	18	133.67	132.88	1.87	10.5	5	90	1.85	26.3
Salbutamol	Female	26	98.68	106.94	2.33	7	6	57	1.58	22.83
Salbutamol	Female	19	103.08	118.11	1.34	8	2.5	60	1.62	22.86
Salbutamol	Male	19	78.62	91.11	1.47	6.5	5	63	1.71	21.55
Salbutamol	Female	20	94.08	94.81	2.14	10.5	5	50	1.62	19.05
Salbutamol	Female	20	92.49	109.9	2.86	3.5	5.5	59	1.65	21.67
Salbutamol	Female	22	92.74	114.39	2.73	7.5	3	78	1.74	25.76
Salbutamol	Female	23	87.16	104.88	0.17	8	4	54	1.62	20.58
failed bronchoscopy	Female	18	107.03	104.56	0.98	4	3.5	57	1.6	22.27
failed bronchoscopy	Female	22	84.21	82.57	2.12	12	5	47	1.52	20.34
failed bronchoscopy	Female	34	97.95	100.75	2.93	9	4	65	1.75	21.22

Group	Sex	Age	% pred FEV ₁	% pred FVC	PC ₂₀ (mg/ml)	Spt (HDM)	Spt (Hist)	Wt (Kg)	Ht (m)	BMI
failed bronchoscopy	Male	21	95.91	91.41	7.93	12.5	5	67	1.8	20.68
failed bronchoscopy	Female	24	108.63	105.47	7.61	12	5.5	49	1.65	18
failed bronchoscopy	Female	21	99.01	84.22	7.7	3	5	51	1.55	21.23
failed screening	Male	21	92.19	105.5	>8	0	3	60	1.8	18.52
failed screening	Male	22	99.53	115.23	>8	3.5	4	60	1.73	20.05
failed screening	Male	22	97.97	110.48	>8	2	3.5	79	1.77	25.22
failed screening	Male	33	118.58	112.24	>8	8	4.5	70	1.76	22.6
failed screening	Male	26	103.31	105.17	>8	7.5	4	80	1.87	22.88
failed screening	Female	59						168	1.52	72.71
failed screening	Male	21	116.42	115.79	>8	13.5	5.5	82	1.83	24.49
failed screening	Male	26	100.64	110.41	0.78	0	5.5	80	1.83	23.89
failed screening	Male	34	117.58	121.18	>8	7	5	95	1.83	28.37
failed screening	Female	21	94.17	87.21	>8	3.5	5.5	48	1.66	17.42
failed screening	Male	18	100.22	95.4	>8	0	4	63	1.75	20.57
failed screening	Female	20	110.25	110.63	>8	0	4	54	1.6	21.09
failed screening	Male	19	117	102.27	>8	4	5	73	1.75	23.84
failed screening	Female	18	95.7	96.43	>8	2.5	3.5	57	1.67	20.44
failed screening	Male	19	115.36		>8	2.5	5	83	1.92	22.52
failed screening	Female	20			>8			86	1.55	35.8
failed screening	Male	21	104.74	100.55	>8	0	2	67	1.8	20.68

Group	Sex	Age	% pred FEV ₁	% pred FVC	PC ₂₀ (mg/ml)	Spt (HDM)	Spt (Hist)	Wt (Kg)	Ht (m)	BMI
failed screening	Female	20	105.33	104.9	>8	2.5	4.5	60	1.6	23.44
failed screening	Female	19	108.99	110.12	>8	2.5	3	74	1.67	26.53
failed screening	Male	20	99.38	95.99	>8	0	5	75	1.83	22.4
failed screening	Female	20				0	3.5	54	1.6	21.09
failed screening	Female	28	117.6	122.66	>8	0	4.5	70	1.75	22.86
failed screening	Female	19	92.77	88.82	>8	2.5	4.5	70	1.8	21.6
failed screening	Male	34	101.83	102.41	>8	7.5	5.5	74	1.7	25.61

Group = group randomised to, or point at which dropped out of study. Pred = predicted, PC₂₀ = PC₂₀ to methacholine (mg/ml), Spt = Skin Prick Test wheal diameter to House Dust Mite (HDM) or histamine (Hist). BMI = Body Mass Index, Ht = Height in metres.

8.4 Characteristics of volunteers in RELM β study (Immunohistochemistry and BAL)

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	ACQ score	ics dose
bg183	healthy	male	36	128.0	17.00	0.00	0.00
ds029	healthy	male	64	89.80	17.00	0.29	0.00
ds031b	healthy	male	19	106.40	17.00	0.00	0.00
ds032i	healthy	male	41	90.90	17.00	0.00	0.00
ds035	healthy	female	19	114.60	17.00	0.00	0.00
ds037	healthy	male	23	105.30	17.00	0.00	0.00
ds040	healthy	male	44	107.90	17.00	0.00	0.00
ds043i	healthy	male	19	87.10	17.00	0.00	0.00
ds056i	healthy	male	20	102.40	17.00	0.00	0.00
ds058i	healthy	female	21	104.00	17.00	0.00	0.00
vk001	healthy	male	21	101.00	17.00	0.00	0.00
vk008	healthy	female	39	94.5	17.00	0.00	0.00

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	ACQ score	ics dose
vk017	healthy	female	23	104.5	17.00	0.00	0.00
CG 1	mild	female	26	98.68	2.33	0.00	0.00
CG 11	mild	female	33	131.49	1.32	0.00	0.00
CG 14	mild	male	23	92.79	0.87	0.14	0.00
CG 15	mild	female	24	115.80	5.04	2.86	0.00
CG 16	mild	female	22	92.74	2.73	0.14	0.00
CG 17	mild	female	25	85.17	1.93	0.71	0.00
CG 19	mild	female	23	107.02	0.81	0.43	0.00
CG 25	mild	male	56	98.07	2.35	1.00	0.00
CG 26	mild	female	21	88.56	0.07	4.14	0.00
CG 3	mild	male	23	95.42	1.59	0.14	0.00
CG 30	mild	male	21	110.88	5.46	0.14	0.00
CG 32	mild	female	22	93.02	0.14	0.57	0.00
CG 35	mild	female	40	98.28	0.30	0.00	0.00

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	ACQ score	ics dose
CG 36	mild	male	23	114.74	6.66	1.29	0.00
CG 38	mild	female	18	99.18	3.69	0.00	0.00
CG 4	mild	male	26	81.61	0.06	2.29	0.00
CG 42	mild	female	30	96.89	0.53	0.57	0.00
CG 5	mild	female	22	91.69	0.45	0.57	0.00
CG 6	mild	female	22	107.53	2.93	0.57	0.00
CG 9	mild	female	22	70.57	5.28	0.71	0.00
ds010	mild	female	18	106.80	2.46	4.71	0.00
ds057	mild	female	37	101.70	1.24	0.28	0.00
bg010	severe	female	50	39.00		4.71	2000.00
bg088	severe	male	60	70.00		2.43	1280.00
bg110	severe	female	44	82.00		3.57	1600.00
bg113	severe	male	56	70.00		2.86	3600.00
bg114	severe	female	60	100.00		1.86	3000.00

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	ACQ score	ics dose
bg128	severe	female	62	87.00		0.57	2000.00
bg129	severe	femle	21	60.00			2000.00
bg132	severe	female	22	113.00		2.71	1200.00
bg135	severe	male	46	35.00		2.71	1600.00
bg144	severe	female	44	89.00			1600.00
bg158	severe	male	58	65.00			2000.00
bg172	severe	female	35	80.00		1.86	1400.00
bg173	severe	female	32	78.60		3.57	2380.00
bg178	severe	female	55	19.80		3.28	1600.00
bg224	severe	male	58	64.90			1600.00
ds005	severe	female	59	68.00		0.57	2800.00
ds008	severe	female	24	70.10	17.00	3.57	4000.00
ds015	severe	female	57	118.60		2.86	2000.00
ds020	severe	female	63	77.80	17.00	3.29	1280.00
ds021	severe	female	59	35.60		3.86	2000.00
ds030	severe	female	45	47.70		2.29	1800.00
ds075	severe	male	28	83.00		6.28	2800.00
ds079	severe	male	61				1000.00

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	ACQ score	ics dose
ds083	severe	female	51	76.00		1.39	2400.00
ds33	severe	male	17	71.25		3.71	3000.00

Pred = predicted, PC₂₀ = PC₂₀ to methacholine (mg/ml), ACQ = asthma quality of life questionnaire, ics = inhaled corticosteroid. ics dose converted to equivalent dose of beclomethasone.

8.5 Characteristics of volunteers in RELM β study (qPCR analysis).

Subject	Subject Group	Age	Gender	BTS Score	Atopic	% pred FEV ₁
BG183	HC	36	M	0	Yes	>100
BG227	HC	55	F	0	No	>100
BG228	HC	31	F	0	Yes	>100
DS043	HC	19	M	0	No	87
DS044	HC	24	M	0	Yes	>100
DS050	HC	27	F	0	Yes	83
DS056	HC	20	M	0	Yes	>100
DS058	HC	21	F	0	No	>100
DS063	HC	24	F	0	No	99
DS064	HC	19	M	0	No	>100
DS065	HC	19	M	0	No	>100
DS066	HC	19	M	0	No	96
DS067	HC	21	M	0	No	83
DS074	HC	18	M	0	No	>100
DS078	HC	54	F	0	No	>100
DS080	HC	19	M	0	No	>100
DS081	HC	22	F	0	No	95
DS087	HC	20	F	0	No	97
DS045	MA	64	F	2	Yes	70

Subject	Subject Group	Age	Gender	BTS Score	Atopic	% pred FEV ₁
DS046	MA	36	F	3	Yes	88
DS047	MA	27	F	1	Yes	>100
DS051	MA	34	F	1	Yes	51
DS052	MA	36	F	1	Yes	96
DS053	MA	42	M	3	No	69
DS057	MA	18	F	1	No	>100
BG010	SA	48	F	5	Yes	39
BG088	SA	58	M	5	Yes	70
BG110	SA	42	F	5	No	82
BG113	SA	54	M	5	No	70
BG114	SA	57	F	4	Yes	>100
BG128	SA	60	F	5	No	87
BG129	SA	21	F	5	No	60
BG132	SA	20	F	5	Yes	>100
BG135	SA	44	M	4	Yes	35
BG144	SA	46	F	4	Yes	89
BG157	SA	40	F	4	Yes	80
BG158	SA	56	M	5	No	65
BG169	SA	46	F	5	Yes	91
BG170	SA	28	M	4	Yes	34
BG172	SA	34	F	5	Not Recorded	80
BG173	SA	32	F	5	Yes	79
BG178	SA	54	F	5	Yes	20

Subject	Subject Group	Age	Gender	BTS Score	Atopic	% pred FEV ₁
BG180	SA	51	F	5	No	30
BG189	SA	40	F	4	No	>100
BG221	SA	69	F	4	No	>100
BG224	SA	57	M	4	Yes	65
BG225	SA	50	F	5	Yes	85
DS069	SA	28	F	4	Yes	64
DS075	SA	28	M	4	Yes	Not recorded
DS077	SA	45	F	5	No	83
DS079	SA	61	M	5	No	Not recorded
DS083	SA	51	F	4	Yes	76

HC - healthy controls, MA - mild asthma, SA - severe asthma, M - male, F - female, BTS score - treatment level on step wise BTS treatment schedule, % Pred FEV₁ - percentage of predicted FEV₁.

8.6 Characteristics of volunteers used in *in vitro* study.

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	reversibility %	ics dose
VK016	Mild asthma	male	36	103.1	17.00	12	0
DS076	Healthy	female	21	104.0	17.00	0	0
CG75	Mild asthma	female	20	92.7	2.73	1	0
DS081	Healthy	female	22	83.0	17.00	1	0
CG81	Mild asthma	female	20	94.1	2.14	11	0
CG82	Mild asthma	female	20	92.5	2.86	9	0
CG83	Mild asthma	female	22	92.7	2.73	16	0
DS87	Healthy	female	20	101.0	17.00	4	0
DS89	Healthy	female	20	93.0	17.00	3	0
KGF02	Mod. asthma	male	47	63.8	0.4	33	500
DS92	Healthy	female	19	77.0	17.00	7	0
CG84	Mild asthma	female	23	87.2	0.17	16	0

Volunteer Number	Group	Sex	Age	% pred FEV ₁	PC ₂₀ (mg/ml)	reversibility %	ics dose
DS95	Healthy	female	20	98.0	17.00	3	0
DS97	Healthy	female	20	109.0	17.00	4	0
KGF08	Mod asthma	female	44	86.0	5.19	14	1000
DS99	Healthy	male	36	110.0	17.00	4	0
DS100	Healthy	female	22	102.0	17.00	5	0

% Pred FEV₁ - percentage of predicted FEV₁. ics dose - dose of inhaled corticosteroid in mg (beclomethasone equivalent doses).

8.7 Cell culture media

All culture medium was obtained from Invitrogen, Paisley, UK.

BEGM Complete

500ml BEGM plus

2ml bovine pituitary extract

500µl hydrocortisone

500µl retinoic acid

500µl epidermal growth factor

500µl epinephrine

500µl transferrin

500µl transferrin

500µl gentamicin-amphotericin B

500µl T₃

500µl insulin

supplied as BEGM bullet kit

ALI Culture medium

ALI cultures were grown in medium containing 1:1 mixture of 2x ALI culture medium and DMEM for ALI culture, plus 1µl of retinoic acid per ml of culture medium.

2x ALI culture medium

50ml BEBM

400µl BPE

100µl EGF

100µl Insulin

100µl hydrocortisone

100µl tri iodo thyronine

100µl epinephrin

100µl Transferrin

100µl BSA

DMEM for ALI culture medium

500ml DMEM

5ml non essential amino acids

5ml sodium pyruvate

5ml penicillin / streptomycin

5ml L glutamine

ALI Culture minimal medium

1ml DMEM

1 ml BEBM

2µl Insulin @ 5.7µg/ml

2µl Transferrin @ 5µg/ml

2µl Penicillin (100U/ml) and Streptomycin (100µg/ml)

8.8 Published work

ERS Congress 2009, Abstract number 256534.

Repeated Inhaled Airway allergen challenge model in asthma

C. Grainge, P.H. Howarth

Allergen challenges are used in drug development to explore cellular and physiological response. Repeated allergen exposure in animals is common but in humans only single high dose inhaled allergen challenges are commonly performed. This is unlikely to reflect responses in natural disease; repeated exposure is more likely to alter the airway inflammatory milieu. In upper airways a repeat challenge model in rhinitis is established; this is not the case with lower airways in asthma. We thus aimed to develop, validate and assess safety of a repeated (3 dose) high dose inhaled allergen challenge protocol in asthmatic volunteers.

16 adults with house dust mite (HDM) allergy & mild non-steroid treated asthma were recruited & exposed to inhaled HDM extract on 3 occasions 48 hours apart. In each challenge, inhaled dose was increased until a 15% drop in FEV₁ was achieved. FEV₁ was recorded every 30 minutes up to 10 hours. There were no significant adverse events. Mean %[$\pm$ SD] maximum falls in FEV₁ from 0-120 mins were similar in all 3 challenges (20.7[6.5], 22.5[7.0], 21.8[6.6], p=0.83). Mean FEV₁ falls from 150-600 mins were also similar (20.7[6.5], 24.2[14.1], 26.5[15.0], p=0.18) as were respective areas under the curve (5455[3980], 5881[3712], 6645[4205], p=0.47). There was no difference in the mean dose of allergen units required (100(0.0), 199(382.7), 238(504.3) p=0.66) or in group mean FEV₁ %predicted prior to each challenge (91.1[12.3], 88.7[12.2], 87.7[12.7] p=0.09). Symptoms (p=0.002) & reliever use (p=0.003) were increased in the week of the challenges. Repeated high dose allergen challenge in humans is safe, repeatable and may be of value in evaluation of novel therapies on indices of inflammation and remodelling in asthma

ERS Congress 2009, Abstract number 256544.

Resistin like molecule beta is upregulated in human respiratory epithelium following repeated inhaled allergen challenge

V Dulay, C Grainge, P Howarth.

Resistin like molecule beta (RELMb) is a soluble protein regulated by an IL4/IL5 STAT6 dependent pathway, and has been shown to directly induce collagen deposition and is necessary for the fibrotic reaction to allergen in mice. There have been no studies examining RELMb in humans following inhaled allergen challenge. We aimed to test the hypothesis that RELMb was present in human airways, upregulated by allergen challenge and associated with remodelling. 16 mild asthmatics with demonstrable allergy to house dust mite (HDM) were repeatedly exposed by inhalation to HDM allergen on 3 separate occasions each 48 hours apart. Each was bronchoscoped before the challenges, and 4 days after the last challenge with bronchoalveolar lavage (BAL) and bronchial biopsies being taken at each bronchoscopy. Biopsy samples were examined by immunohistochemistry and the percentage staining of the bronchial epithelium measured in duplicate at each time point. Group mean ($\pm$ SD) BAL eosinophils were elevated following repeated allergen challenge (3.9% (4.5) to 10.3 (12.0) $p=0.005$) as was percentage staining of the epithelium with RELMb (2.8%(2.4) to 4.9%(3.4) $p=0.016$). Basement membrane thickness also increased (7.8 μ m (1.4) to 9.6 (2.3) $p=0.023$). Change in thickness of the basement membrane was however not correlated with change in RELMb staining ($p=0.86$).

We demonstrate that RELMb is present in human airways, localized to epithelial cells and upregulated following a repeated allergen challenge that provokes a prolonged eosinophil response. RELMb upregulation did not, however, correlate with change in basement membrane thickness, as potentially predicted by animal studies.

Grainge C, Howarth PH. Repeated high dose allergen challenge in asthma. CRJ 2010. DOI: 10.1111/j.1752-699X.2010.00212.x

crj_212

The Clinical Respiratory Journal

ORIGINAL ARTICLE

Repeated high-dose inhalation allergen challenge in asthma

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Abstract

Introduction: Inhalation allergen challenge in humans is used to investigate lung pathophysiology and responses to novel therapies. However, the single high-dose allergen challenges that are commonly performed do not mimic repeated symptomatic environmental allergen exposure.

Objectives: To develop and evaluate the safety of a repeated high-dose symptomatic inhalation allergen challenge model.

Methods: Sixteen subjects with atopic asthma were recruited. Each underwent three inhalation allergen challenges using house dust mite (*Dermatophagoides pteronyssinus*) antigen at 48-h intervals with a target of symptom induction and an early asthmatic reaction fall in FEV₁ of 15% from baseline.

Results: All of the subjects completed the three-challenge protocol and the target immediate airway bronchoconstrictor response was achieved in all the subjects at all challenges. There were no adverse events recorded. The early asthmatic reaction was similar for the three challenges whether measured as mean maximal fall in FEV₁ or mean area under the curve. The late asthmatic reaction was also similar over the three challenges with no evidence of priming or desensitisation. Symptom scores and reliever medication use significantly increased over the time of the challenges. Baseline lung function and reversibility was unchanged 4 days after the last challenge.

Conclusion: We demonstrated that repeated high-dose inhaled house dust mite allergen challenge in human volunteers with mild asthma is safe, repeatable and acceptable. This allows the use of this model in further studies focused on the understanding the pathophysiology of allergen induced asthma and the impact of therapeutic interventions.

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Key words

adult – allergens – antigens :
dermatophagoides – asthma – bronchial
provocation tests – humans

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Authorship

Both authors designed and performed the study, analysed the data and wrote the manuscript.

Ethics

The study was performed with the approval of the Southampton and South West Hampshire Research Ethics Committee (A) (reference 08/H0502/5) and written informed consent was obtained from all participants prior to enrolment.

Conflict of interest

The authors have no conflicts of interest to declare.

Introduction

Inhalation allergen challenge is used in asthma to investigate the impact of novel interventions on lung function and indices of airway inflammation (1, 2). Standardly, this is composed of a single allergen challenge targeted to achieve an FEV₁ drop of between 15 and 30% from baseline (2–6), and although these challenges are safe and repeatable (7) and correlate well

with single environmental exposures (8), there is debate as to whether they reflect and thus model repeated allergen exposure in the wider environment. Allergen challenge in the environment is likely to be repeated rather than single dose and may or may not produce repeated symptoms. To address the situation of repeated but essentially asymptomatic environmental allergen exposure, a repeated low-dose allergen challenge model has been developed in which there is

crj_212

Repeated high-dose inhalation allergen challenge in asthma

Grainge and Howarth

no target FEV₁ drop, rather a lower dose of allergen is repeatedly administered with the aim of inducing more sustained airway inflammation (9). This model has been used to investigate the effects of pretreatment (10) but does not consistently induce eosinophilic airway inflammatory changes. The situation of repeated symptomatic environmental allergen exposure has not been addressed experimentally in human subjects; by contrast, preclinical animal challenge protocols, designed to investigate novel asthma medications, use repeated high-dose allergen exposure. These models demonstrate sustained airway eosinophilic inflammation and structural airway remodelling changes (11–14). There is currently no direct human model that relates to these animal-based protocols, possibly on account of concerns over safety.

Current human allergen challenge protocols, both single high-dose and repeated low-dose models are useful in understanding the basis of disease, but have two particular insufficiencies that could be addressed by a repeated high-dose challenge model. Firstly, individuals may be exposed repeatedly to symptomatic doses of allergen from the environment, and secondly, the animal models, which have been so useful in developing our understanding of the disease, have often been developed with repeated allergen challenge (15); these models should be validated against human responses in order to support their previous findings and future use.

Thus, to evaluate the safety of repeated high-dose inhalation allergen challenge, prior to investigating the potential of such a model for the investigation of the mechanisms of allergen-induced airway remodelling in asthma, we undertook a repeated inhalation allergen challenge protocol. This was performed in volunteers with mild asthma and involved three allergen challenges each separated by 48 h, with assessment of lung function and symptom outcomes.

Here, we describe a safe, repeatable and clinically acceptable repeated allergen challenge protocol involving three allergen challenges each separated by 48 h. This challenge protocol will be useful in establishing human correlations with repeated allergen exposures in laboratory animals and also determining how repeated environmental allergen challenge differs from the established single allergen challenge model.

Materials and methods

Subjects

Sixteen subjects (four male) (mean age 24.1 years, range 21–40) with physician-diagnosed atopic asthma

were recruited by local advertisement and from a database of volunteers held by the department. The study was approved by the local research ethics committee, and all the subjects provided written informed consent. All of the participants were non-smokers, had an FEV₁ of >70% of predicted (16) and were only receiving treatment with short-acting inhaled β_2 agonists as needed. Additionally, all of the participants had a PC₂₀ to methacholine of <8 mg/mL [median value (interquartile range, IQR) 1.10 (1.76) mg/mL] and were skin prick test positive to house dust mite (HDM) (*Dermatophagoides pteronyssinus*) [median (IQR) wheal diameter 7.25 (2.9) mm]. If subjects were also pollen sensitive they were studied outside the relevant period of pollination in the winter months.

FEV₁ measurements and reversibility

Following screening, and at least 2 weeks prior to the repeated allergen challenge, the subjects underwent reversibility testing using 2.5 mg of salbutamol administered by an air-driven nebuliser (Respiromics, π , UK). This test was repeated 4 days after the last allergen challenge.

Allergen challenge

Three allergen challenges were performed at 48-h intervals. Fresh solutions of freeze-dried HDM allergen extract (Diagenics Ltd, Milton Keynes, UK) were made up with 0.9% saline to a concentration of 5000 U/mL. The solution was administered using a breath activated dosimeter connected to a controlling computer (APS pro, Jaeger, π , Germany) (7).

After measurement of baseline spirometry (FEV₁ and FVC) and providing these values were, respectively, $\geq 70\%$ and $\geq 80\%$ predicted, the subject underwent the challenge. Initially, 0.1 mg of 0.9% saline was administered with three recordings of FEV₁ each at 5 and 10 min post-challenge, with the best reading from each time point used for analysis. The lower of these readings at 5 or 10 min was then used as the baseline for further analysis. Initial dosing of allergen was then with 10 U. FEV₁ was recorded at 5, 10 and 15 min, as mentioned earlier. Further allergen was then incrementally administered at 15 min intervals, using 40, 160, 720, 2100 and 5000 U, with spirometric recording post-challenge, as mentioned earlier, until a fall in FEV₁ of $\geq 15\%$ compared with post-saline reference was obtained. If FEV₁ fell by between 10 and 15%, the previous dose was repeated rather than incrementing further. The cumulative dose required was recorded as

the PD₁₅. FEV₁ was then measured at 20, 30, 45 and 60 min, then at 30 min intervals up to 10 h.

On subsequent allergen challenges, the aforementioned protocol was followed, except that half the PD₁₅ from the previous challenge was used as the initial dose to account for possible sensitisation. If this did not cause a $\geq 15\%$ decrease in FEV₁, the same dose was administered again (giving the total PD₁₅ from the previous challenge). If a FEV₁ drop of $\geq 15\%$ was not achieved, the dose was increased according to the previously detailed protocol.

Symptom diary

The subjects completed a 14-day symptom diary morning; the first challenge day was on day eight, allowing comparison for the week before challenge and the week during the repeated challenges. The minimum score, associated with no symptoms, was zero; the maximum score possible was 30. Total daily puffs of inhaled short acting β_2 agonist were also recorded.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Mac except for area under the curve (AUC) analysis, which was performed using Prism 3.0 for Windows. All repeated challenge lung function and allergen dose measures were analysed using Friedman's test, while symptom scores and other paired data were analysed using Wilcoxon signed-rank test. A *P* value of <0.05 was taken as statistically significant.

Results

Safety

There were no serious adverse events such as significant worsening of asthma requiring hospital admission nor was there any requirement for the introduction of inhaled or oral steroids. All of the subjects completed the three-challenge protocol.

Dose of provocation

In order to detect priming or desensitisation, the dose of provocation required to induce the target early asthmatic reaction (EAR) was recorded and expressed as a percentage of the dose required in challenge 1. There was no significant change in the doses required at the three challenges; median (interquartile range) doses administered were 100% (0.0), 100 (42.5) and 100 (66.0) (*P* = 0.66).

Symptom score and reliever use

Mean daily symptom score and reliever use in the week of the challenges increased significantly compared with the preceding week. Mean (SD) daily symptom score increased from 0.9 (1.6) to 3.4 (2.7) (*P* = 0.002), while reliever use increased from a daily mean (SD) of 0.2 (0.4) to 1.7 (1.9) (*P* = 0.003) (Table 1).

Magnitude of responses

The required immediate airway bronchoconstrictor response was achieved in all subjects at all challenges. The EAR, defined as the fall in FEV₁ from 0 to 120 min following challenge, was similar for the three challenges whether measured as mean (SD) maximal fall in FEV₁ (20.7% (6.5), 22.5% (7.0), 21.8% (6.6), *P* = 0.83) or as mean (SD) AUC for that time period (1 161 379), 1302 (507), 1510 (685), *P* = 0.07). The late asthmatic reaction (LAR) was defined as the FEV₁ fall from post-saline baseline from 150 to 600 min post-challenge and was also similar, when measured as the mean maximal FEV₁ fall (20.7% (6.5), 24.2% (14.1), 26.5% (15.0), *P* = 0.18) or as the AUC for that time period (54 553 980), 5881 (3712), 6645 (4205), *P* = 0.47) (Table 2 and Fig. 1).

FEV₁ prior to repeated challenges and airway reversibility

At screening, mean (SD) percentage predicted FEV₁ was 94.3% (14.2). Mean (SD) percentage predicted

Table 1. Symptom score and reliever use in week prior to and week of repeated inhaled house dust mite allergen challenges

	Week before repeated challenges	Week of repeated challenges	<i>P</i> value
daily symptom score	0.9 (1.6)	3.4 (2.7)	0.002
daily reliever use	0.2 (0.4)	1.7 (1.9)	0.003

P values calculated by Wilcoxon signed-rank test. Symptom scores in arbitrary units. Values are means with SD in parentheses.

crj_212

Repeated high-dose inhalation allergen challenge in asthma

Grainge and Howarth

Table 2. Dose of provocation required, changes in FEV₁ and area under the curve for three repeated inhaled house dust mite allergen challenges at 48-h intervals

	Allergen challenge number			P value
	1	2	3	
Dose of provocation	100 (0.0)	100 (42.5)	100 (66.0)	0.66
Baseline FEV ₁	91.1 (12.3)	88.7 (12.2)	87.7 (12.7)	0.09
EAR FEV ₁	20.7 (6.5)	22.5 (7.0)	21.8 (6.6)	0.83
EAR AUC	1161 (379)	1302 (507)	1510 (685)	0.07
LAR FEV ₁	22.8 (13.6)	24.2 (14.1)	26.5 (15.0)	0.18
LAR AUC	5455 (3980)	5881 (3712)	6645 (4205)	0.47

P values are calculated using Friedman's test. Values are means with SD in parentheses, except dose, which is median, with interquartile range in parentheses. EAR, early asthmatic reaction (0 to 120 min); LAR, late asthmatic reaction (150 to 600 min); Baseline FEV₁, percentage predicted FEV₁ prior to saline inhalation; EAR/LAR FEV₁, maximal change in FEV₁ from post saline baseline; AUC, area under the curve.

FEV₁ 4 days after final challenge was 95.5% (14.1), which was not different from screening ($P=0.53$). There was no significant change in the baseline pre-challenge FEV₁ with repeated allergen challenge. The mean (SD) percentage predicted baseline FEV₁ prior to each repeated challenge was 91.1% (12.3), 88.7% (12.2) and 87.7% (12.7), respectively ($P=0.09$) (Table 3).

Mean (SD) FEV₁ reversibility to 2.5 mg nebulised salbutamol was similar prior to and following repeated challenge, at 7.5% (5.9) prior to and 9.9% (6.4) after challenges ($P=0.12$) (Table 3).

Discussion

This study has demonstrated that repeated high-dose inhaled HDM allergen challenge in human volunteers with mild asthma is safe, repeatable and acceptable.

Previously, only limited information has been available regarding repeated allergen challenge in human asthmatic volunteers and none of it sufficiently strong to give confidence in the safety of a repeated high-dose allergen protocol. One small study reported the effects of two challenges in eight volunteers (17). In this study, inhalation allergen challenge, targeted to produce a fall in FEV₁ of 15% from baseline, was repeated after an

interval of 48 h. No changes in the intensity of the EAR and LAR were identified. This study, additional to its sample size, was, however, limited in that the study population was mixed, in that different allergens (HDM and grass pollen) were used in different individuals. It has recently been demonstrated that these allergens have a different propensity to induce a LAR response (18), and this has implications for severity of response. Similarly, one other study that evaluated the impact of four successive allergen challenge days on priming or desensitisation (19) recruited those with varying allergic sensitivity, with the predominant incorporation of ragweed pollen sensitive subjects. Although this study found no deleterious effect, it only included two subjects who had late reactions as defined solely by symptom reporting with no spirometric measures.

In our study, all of the subjects were studied with the same HDM challenge, and despite only having approximately a mean 20% fall in FEV₁ during the immediate reaction, had a mean late reaction fall in FEV₁ of 20%–30%, with 14 of the 16 subjects having a late reaction as defined by a fall in FEV₁ of $\geq 15\%$ from baseline at some time between 150 and 600 min post-challenge. The LAR has been linked to eosinophilic airway inflammation and enhanced bronchial hyperresponsiveness (3). The late reaction is thus considered better repre-

Table 3. Percentage predicted FEV₁ and reversibility prior to and following repeated allergen challenge

	Prior to repeated challenges	Four days after repeated challenges	P value
Percentage predicted FEV ₁	94.3 (14.2)	95.5 (14.1)	0.53
Percentage FEV ₁ reversibility	7.5 (5.9)	9.9 (6.4)	0.12

P values calculated by Wilcoxon signed-rank test. Reversibility measured using 2.5 mg nebulised salbutamol. Values are means with SD in parentheses.

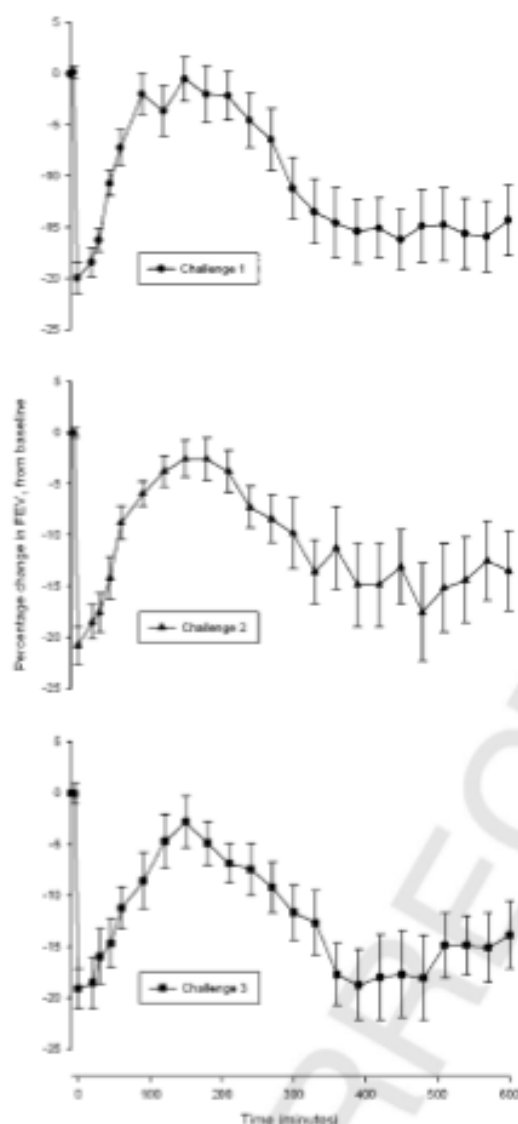


Figure 1. Mean percentage change in FEV₁ from baseline over time following three inhaled house dust mite allergen challenges at 48-h intervals. Bars = standard error of the mean.

sentative of clinical asthma, and by selecting HDM-sensitive subjects, we have biased our study to selecting those who may potentially deteriorate with repeated allergen challenge. Our study did not specifically investigate the impact of repeated allergen challenge on bronchial reactivity in case reactivity measures them-

selves had some confounding impact. Similarly, we did not induce sputum to assess airway inflammatory cell changes and other markers of ongoing airway inflammation. As the safety of this model has been established, it will now be possible to assess the impact of this repeated inhalation allergen challenge protocol on these outcome measures and investigate their relationship to the change in symptoms.

The repeated allergen challenge induced an increase in symptoms and an increased need for short-acting beta-agonist use. There was, however, no indication of priming or indeed of desensitisation in that there was no significant difference in allergen dose administered in any of the three separate challenges. Our safety evaluation was additionally based on lung function measures. No significant difference was evident on the third challenge day in either baseline lung function pre-challenge, early or late lung function changes in response to the challenge or in baseline lung function and reversibility 4 days post-challenge. There was a trend towards a greater AUC for the early reaction ($P = 0.07$) with repeated challenge so if a study protocol was planned that involved more than three repeated challenges, this trend towards a reduction in FEV₁ could become both statistically and clinically significant and would need evaluating. However, this study has shown that three repeated allergen challenges in human asthma volunteers designed to produce a mean early fall in FEV₁ of 15% is safe and repeatable. This thus allows the use of this model in further understanding the pathophysiology of allergen-induced asthma and the impact of therapeutic intervention such as in the development of new asthma treatments or the assessment of efficacy of immunotherapy.

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crj_212

Repeated high-dose inhalation allergen challenge in asthma

Grainge and Howarth

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