Reduced corticosteroid sensitivity in smokers with asthma: potential mechanisms and treatment

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ABSTRACT

Smokers with asthma display reduced responses to both inhaled and oral corticosteroids with associated increased symptoms, accelerated decline in lung function and increased use of health care services. Little work has been undertaken to address the possible causes of this reduced response and to find effective replacement therapies. Therefore this thesis was carried out with the aim of identifying potential mechanisms and new therapies for this group.

The oral bronchodilator theophylline has been suggested as a treatment for corticosteroid insensitivity due to its ability to increase HDAC activity *in-vitro*. I undertook an exploratory proof of concept clinical trial based on the hypothesis that low dose theophylline would restore corticosteroid sensitivity in smokers with asthma through theophylline induced recovery of HDAC activity. Low dose oral theophylline added to inhaled corticosteroid increased pre-bronchodilator lung function and reduced symptoms of asthma whilst low dose theophylline given alone reduced symptoms but had no effect on pre-bronchodilator lung function. This research provides a foundation for future studies designed to examine the efficacy of theophylline in smokers with asthma.

Agonists of the nuclear hormone receptor peroxisome proliferator activated receptor-γ (PPARγ) have been demonstrated to be effective at reducing inflammation in both *in-vitro* and animal models of asthma. Therefore to examine the hypothesis that PPARγ stimulation would reduce the inflammation present in smokers with asthma I undertook an exploratory, proof of concept clinical trial using the PPARγ agonist rosiglitazone. Treatment with rosiglitazone was associated with a trend to improvement in FEV_1 and improvement in a marker of small airway lung function and as such may provide an alternative treatment for small airways obstruction in conditions such as asthma and chronic obstructive airways disease. This trial will enable powering of future confirmatory studies.

Altered cytokine profiles, specifically the combination of increased interleukin (IL)-2 and 4, are observed in asthmatic subjects with corticosteroid insensitivity. Based on this work I examined the hypothesis that the altered response to

corticosteroids in smokers with asthma was associated with an altered cytokine milieu including raised levels of IL-2 and 4. Smokers with asthma, characterised as corticosteroid resistant by oral corticosteroid trial, demonstrated significantly raised sputum supernatant IL-6 levels and raised levels of a number of other sputum cytokines compared to non smokers with asthma. This altered phenotype suggests cigarette smoking in asthma may be associated with a deviation to Th1 mediated inflammation and could provide an explanation for the reduced corticosteroid response of smokers with asthma. The cell type/s responsible for both this shift in immunological phenotype and production of increased levels of sputum cytokines is unclear and will require further study.

Previous *in-vitro* and *in-vivo* research has identified altered histone acetylation patterns in subjects with relative corticosteroid resistance. Therefore I examined the hypothesis that smokers with asthma displayed reduced responses to corticosteroids as a result of a cigarette smoke induced reduction in histone de-acetylase (HDAC) activity. Smokers with asthma provided sputum macrophages and blood for peripheral blood borne monocytes to examine total HDAC activity. Sputum and blood macrophage total HDAC activity was equivalent in smokers and non-smokers with asthma. Therefore reduced blood total HDAC activity does not appear to explain the altered corticosteroid response in this group. However the number of sputum macrophages obtained may have been too low to allow conclusive examination of this endpoint. Another consideration is that contamination of the sample due to the technique used may be altering the signal obtained. Further work either through modification of sputum induction techniques to increase macrophage number or bronchoscopic sampling is required to conclusively address the role of alveolar macrophage HDAC activity in the reduced corticosteroid response displayed by smokers with asthma.

Exhaled nitric oxide has been exploited as a useful exploratory and confirmatory endpoint in asthma. However exhaled nitric oxide, measured using standard flow rates and methodology, is unhelpful in smokers with asthma as cigarette smoking is associated with a marked reduction in exhaled nitric oxide levels in the majority of subjects. Recent research has demonstrated that measurement of exhaled nitric oxide at multiple flow rates followed by mathematical modelling reveals increased levels of alveolar nitric oxide that were unaltered by current smoking. Therefore to examine the hypothesis that smokers with asthma display

altered levels of alveolar nitric oxide and flow independent parameters compared to non-smokers with asthma I carried out a cross-sectional study. Alveolar nitric oxide, determined by linear modelling, was significantly reduced in smokers with asthma compared to non smokers with asthma. The concentrations observed were within the range for normal subjects and therefore this method does not overcome the problems inherent in measuring exhaled nitric oxide at standard flows. The use of non-linear modelling did demonstrate parity between smokers and non-smokers with asthma for alveolar nitric oxide. Nitric oxide flux was lower in smokers with asthma when derived by both linear and non-linear modelling and displayed sensitivity to oral corticosteroids. Therefore nitric oxide flux is worthy of further investigation as an exploratory endpoint in smokers with asthma.

In conclusion treatment of smokers with asthma with low dose theophylline alone, the combination of low dose theophylline and inhaled corticosteroid and the PPARγ agonist rosiglitazone was associated with clinical improvements and further clinical trials to assess the role for these treatments in the management of smokers with asthma are justified. Smokers with asthma display an altered sputum cytokine profile with raised levels of the proinflammatory cytokine IL-6, equivalent blood total HDAC activity and reduced alveolar nitric oxide compared to non-smokers with asthma. Sputum HDAC activity requires further development before it can be confidently employed as a method of assessing total pulmonary HDAC activity.

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DECLARATION

I am the sole author of this thesis and have personally consulted all the references listed. I was solely responsible for the day to day running of both projects and I recruited, screened, obtained informed consent and performed randomisation of all the patients and the majority of follow up visits. The remainder of follow up visits were performed by Maureen Brannigan & Joyce Thompson with occasional help from Rekha Chaudhuri and Jane Lafferty (All employees of Asthma Research Unit, University of Glasgow).

I generated the majority of approach letters and performed the majority of GP practice searches with occasional help from Joyce Thompson, Maureen Brannigan, June McGill & Janice Reid (both West Node, SPCRN).

Hiran Cooray (GSK Discovery Medicine Section), Colin Rodden and Anne Watt (Gartnavel General Pharmacy) provided medication packs for the theophylline and rosiglitazone study. Colin Rodden and Anne Watt provided dexamethasone packs for the corticosteroid sensitivity study.

Iona Donnelly, Lisa Jolly and Poh Lin Winn (GBRC, University of Glasgow) prepared the sputum, supernatant and blood samples and performed sputum cell counts, supernatant and plasma Luminex and the sputum RANTES ELISA. I performed the sputum IL-6 ELISA with help from Lisa Jolly and Grace Murphy (GBRC, University of Glasgow). The sputum IL-8 and MPO ELISAs were performed by GSK Discovery Medicine Section.

Kazuhiro Ito and Kenichi Akashi (NHLI Imperial College) performed the HDAC activity assay.

I performed the data entry and the majority of the analysis for the theophylline and rosiglitazone study. Lisa Sweeney (GSK Discovery Medicine Section) performed the lung function analysis. I also performed the data entry for the trial examining corticosteroid responses. Dr Chris Weir (Robertson Centre for Biostatistics, University of Glasgow) performed the majority of the analysis in the trial examining corticosteroid responses in subjects with asthma with supplementary analyses performed by myself where required. This thesis has not been previously been submitted for a higher degree.

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ABBREVIATIONS

ACQ	asthma control questionnaire
ADP	adenosine diphosphate
AFU	arbitrary fluorescence units
AQLQ	asthma quality of life questionnaire
AP1	activator protein 1
ATP	adenosine trisphosphate
BAL	bronchoalveolar lavage
BMI	body mass index
C_{alv}	alveolar nitric oxide concentration
C_{aw}	airway wall concentration of nitric oxide
CCL	chemokine with C-C N terminal motif
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
COPD	chronic obstructive pulmonary disease
CXC	chemokine with C-X-C N terminal motif
D_{aw}	airway wall nitric oxide diffusion
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBC	exhaled breath condensate
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
FEV_1	forced expiratory volume in the first second
FE_{NO}	exhaled nitric oxide concentration
FE_{NO50}	exhaled nitric oxide concentration at flow rate of 50ml/sec
FEF_{25-75}	forced mid-expiratory flow rate
FEF_{75}	forced expiratory flow at 75% of FVC
FVC	forced vital capacity
GM-CSF	granulocyte/monocyte-colony stimulating factor
GR	glucocorticoid receptor
GRα	glucocorticoid receptor alpha
GRß	glucocorticoid receptor beta
HAT	histone acetyltransferase
HDAC	histone de-acetylase

H_2O_2	hydrogen peroxide
ICC	intraclass correlation
ICS	inhaled corticosteroid
IFN-α	interferon-α
IFN-γ	interferon-γ
IgE	immunoglobulin E
IκB	inhibitor of κB
IL	interleukin
IL-1RA	IL1-receptor antagonist
IL-2R	IL-2 receptor
IP-10	Interferon-inducible Protein of 10 kDa (aka CXCL10)
iNOS	inducible nitric oxide synthase
IQR	interquartile range
IU	international units
Jaw	airway wall nitric oxide flux
J'aw	maximal airway wall nitric oxide flux
JNK	c-Jun N-terminal kinase
kDa	kilodalton
kg/m^2	kilograms/metres squared
LABA	long acting β$_2$ receptor agonist
L/min	litres per minute
Log	logarithm
LPS	lipopolysaccharide
MAPK	mitogen associated protein kinase
MCP-1	Monocyte Chemotactic Protein 1
MEK	MAPK kinase
MIG	Monokine Induced by IFN-γ (aka CXC9)
MIP-1α	Monocyte Inflammatory Protein 1α (aka CCL3)
MIP-1ß	Monocyte Inflammatory Protein 1ß (aka CCL4)
mRNA	messenger ribonucleic acid
NCoR	nuclear receptor co-repressor
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	neuronal nitric oxide synthase
NO	nitric oxide concentration

NSAID	non-steroidal anti-inflammatory drug
PC_{20}	provocative concentration of methacholine causing a 20% fall in FEV_1
PDE	phosphodiesterase
PEF	peak expiratory flow
PBMC	peripheral blood borne monocyte
PI3K	phosphatidylinositol 3 kinase
pl/s	picolitres per second
pl/s/ppb	picolitres per parts per billion per second
ppb	parts per billion
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator response element
PTEN	phosphatase and tensin homolog deleted on chromosome ten
p38	p38 MAPK
RANTES	regulated upon activation, normal T cell expressed and secreted (aka CCL5)
RNA	ribonucleic acid
RU486	mifepristone
RXR	retinoid X receptor
SAE	serious adverse event
SD	standard deviation
SEM	standard error of the mean
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
STAT	signal transducers and activator of transcription
SUMO	small ubiquitin-like modifier
TGFß	Transforming Growth Factor ß
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
Th17	IL-17 positive T helper cells
TNFα	Tumour Necrosis Factor-α
Treg	Regulatory T lymphocyte
TSA	trichostatin A
V_E	exhalation flow rate
V_{NO}	elimination rate of exhaled nitric oxide

4HNE   4-hydroxynonenal

15d-PGJ$_2$ 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$

95% CI   95% confidence interval

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1. Effect of theophylline plus beclometasone on lung function in smokers with asthma-a pilot study
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1. Exhaled Breath Condensate pH response to oral corticosteroid in smokers, ex-smokers and non-smokers with asthma

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2. Comparison of extended flow nitric oxide parameters in smokers and non-smokers with asthma before and after an oral corticosteroid trial

   Spears M, Weir C, Thompson J, Brannigan M, Lafferty J, Chaudhuri R, Braganza G, Cameron E & Thomson NC.

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3. Smokers with asthma display an altered sputum cytokine profile that is insensitive to short course oral corticosteroid therapy

   Spears M, Donnelly I, Jolly L, Winn PL, Weir C, McSharry C, Chaudhuri R, Thompson J, Brannigan M, Lafferty J & Thomson NC.

   Poster discussion. American Thoracic Society Meeting, San Diego, May 2009

4. Efficacy of two new approaches to treatment of smokers with asthma. Spears M, Donnelly I, Jolly L, Brannigan M, Ito K, McSharry C, Lafferty J, Chaudhuri R, Braganza G, Bareille P, Sweeney L, Adcock IA, Barnes PJ, Wood S & Thomson NC

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5. Efficacy of low dose theophylline and inhaled corticosteroids in smokers with asthma

   Spears M, Donnelly I, Jolly L, Brannigan M, Ito K, McSharry C, Lafferty J, Chaudhuri R, Braganza G, Bareille P, Sweeney L, Adcock IA, Barnes PJ, Wood S & Thomson NC.

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6. Efficacy of the PPARγ agonist rosiglitazone in smokers with asthma.

   Spears M, Donnelly I, Jolly L, Brannigan M, McSharry C, Chaudhuri R, Bareille P, Sweeney L, Lafferty J, Wood S & Thomson NC.

   Poster. American Thoracic Society Meeting, Toronto, May 2008

ORAL PRESENTATIONS OF WORK FROM THESIS

1. Overcoming relative corticosteroid insensitivity and enhancing the treatment response of smokers with asthma.
   November 2008. Celsus meeting, University of Glasgow.

2. PPARγ agonists in asthma-a proof of concept clinical trial.
   August 2008. Divisional Away Day, Division of Immunology Infection & Inflammation, University of Glasgow.

3. Reversing corticosteroid sensitivity in Smokers with Asthma.
   August 2008. Divisional Away Day, Division of Immunology Infection & Inflammation, University of Glasgow.

4. Efficacy of low dose theophylline and inhaled beclometasone and rosiglitazone alone compared to inhaled beclometasone in Smokers with Asthma.
   May 2008. Scottish Society of Experimental Medicine Meeting, Dundee
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5. Therapeutic impact of Rosiglitazone and low dose Theophylline in Smokers with Asthma. April 2008. Scottish Thoracic Society Meeting, Perth

6. Rosiglitazone in Smokers with Asthma; a possible new class of anti-inflammatory treatment? January 2008, SSPC meeting, Carnoustie

7. PPARγ agonists as potential anti-inflammatory agents for Smokers with asthma. August 2006. Divisional Away Day, Division of Immunology Infection & Inflammation, University of Glasgow.

8. Corticosteroid insensitivity in Smokers with Asthma. August 2005. Divisional Away Day, Division of Immunology Infection & Inflammation, University of Glasgow.

1 Introduction

1.1 Asthma

Asthma is a common condition, characterised by variable symptoms of breathlessness, cough and/or wheeze combined with variable airflow obstruction and inflammation (1, 2). A recent report estimated that 300 million people worldwide are currently affected by asthma (3). The prevalence of asthma varies between countries and the UK has one of the highest rates with 5 million people currently receiving treatment (4). Scotland is disproportionately affected as it has the world's highest prevalence in children (35%) and a high proportion of affected adults (18%) (3).

Whilst the majority of patients with asthma can achieve a degree of control with either inhaled corticosteroids, a combination of inhaled steroids and other inhaled or oral therapies there is still a large sub-group who fail to achieve this. Smokers with asthma are normally excluded from clinical trials due to a desire to exclude subjects with chronic obstructive pulmonary disease but recent research demonstrates that smokers with asthma have a poor response to inhaled corticosteroids (5-10) and worse symptoms from asthma as demonstrated by asthma questionnaire (11, 12). Therefore it can be reasonably argued, given the prevalence of smoking in asthma, that smokers with asthma are a large neglected and important group that require further study and new therapies (13). It is against this background that this thesis was undertaken with the aim of understanding and exploring possible mechanisms for improving asthma control in smokers with asthma.

1.2 Smokers with asthma

1.2.1 Prevalence of active smoking in asthma

Despite many years of public health programmes highlighting the negative health effects of smoking this habit is still surprisingly common in asthmatics. Current estimates suggest twenty to thirty five percent of adult asthmatics in Northern

Europe are current smokers and at least half the adult population with asthma are current or ex-cigarette smokers (11, 14, 15). The prevalence of smoking is higher in asthmatics that visit emergency departments with asthma exacerbations (16, 17) or who have died from asthma (18, 19) than general population estimates of current smoking in asthma and the death rate six years after admission to hospital with a near fatal asthma attack is higher amongst smokers compared to non-smokers (20). Even when mild forms of asthma are included in analyses smokers with asthma have worse symptoms compared to matched non smoking asthmatics (11, 12) and smokers with asthma display an accelerated decline in lung function (15, 21). This phenomenon is associated with evidence of an altered response to both inhaled (5-10) and oral corticosteroids (22-24) in smokers with asthma.

1.2.2 Clinical evidence for reduced corticosteroid sensitivity in smokers with asthma

The first prospective randomised controlled trial to demonstrate corticosteroid insensitivity in smokers with asthma compared the effect of three weeks treatment with inhaled fluticasone propionate (1000 mcg daily) to placebo in a cross-over study of 17 smokers and 21 non-smokers with corticosteroid-naïve asthma (7). Non-smokers demonstrated a significant increase in FEV_1, PEF, PC_{20} and a decrease in proportion of sputum eosinophils. This contrasted with the smokers with asthma who did not demonstrate improvements for any of these endpoints.

However a potential criticism of this study was its short duration and therefore another clinical trial addressed this issue (8). Inhaled beclometasone at doses of either 400 mcg or 2000 mcg daily were allocated for 12 weeks to smokers and non-smokers in a double blind randomised controlled fashion. At the conclusion of the trial the non-smoking subjects treated with 400 mcg significantly improved their morning PEF compared to smokers (figure 1.1). This was associated with a reduction in asthma exacerbations and was in contrast to the smokers with asthma who did not demonstrate any significant improvements. However when treated with 2000 mcg inhaled beclometasone there was no significant difference between smokers and non-smokers with asthma. This

result would appear to indicate that higher doses of corticosteroids can produce an equivalent level of lung function response to that produced in non smokers with asthma using standard low doses. However an interaction analysis suggested that the higher dose was not performing significantly better than the lower dose. Therefore further trials using high dose inhaled corticosteroids in smokers with asthma are required to clarify this point.
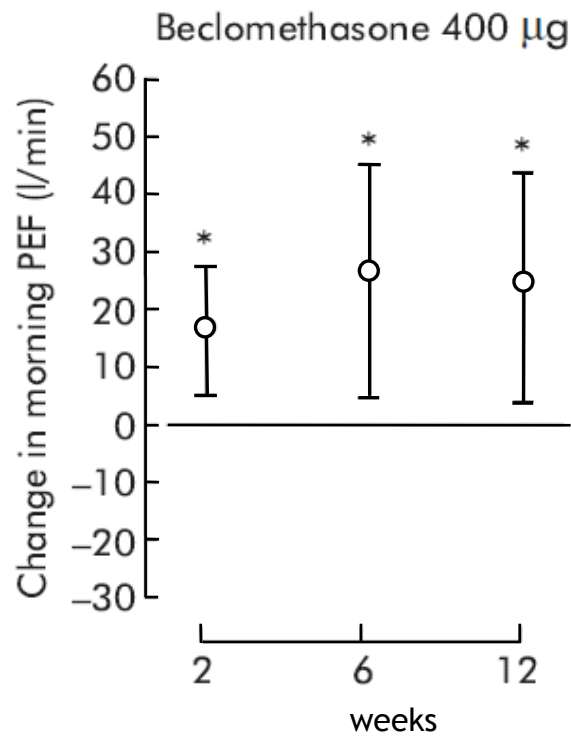
Figure 1.1 Mean (95% CI) change in morning PEF (L/min) in smokers compared to non-smokers with asthma.

Mean change above the line demonstrates greater improvement in non-smokers with asthma. *; p<0.05. Adapted from (20).

A further study carried out by a separate group examined the corticosteroid response of smokers with asthma with lower pack year histories (9). The multi-centre randomised cross-over trial undertaken by the Asthma Clinical Research Network in the USA examined the treatment response of a group of smokers with asthma (compared to non-smokers with asthma) to eight weeks of 400mcg inhaled CFC free beclometasone ('QVAR[TM]') or montelukast. The mean pack year history in the smokers was 7 years. Treatment with inhaled beclometasone improved FEV_1, PEF and PC_{20} in the non-smokers but not in the smokers.

A post hoc analysis of a large management trial designed to achieve total control of asthma through the use of inhaled fluticasone and the combination of fluticasone and salmeterol for one year confirmed and strengthened this finding of reduced response to inhaled corticosteroids in subjects with low pack year histories (10, 25). Logistic regression analysis demonstrated that smokers with asthma with less than ten pack years had an odds ratio for poorly controlled asthma of 2.8 (95% CI 2.0-3.7) despite treatment with inhaled fluticasone (23). At the conclusion of the trial only 40% of smokers had achieved well controlled asthma on inhaled fluticasone alone compared to 63% of non-smokers (10).

The lung function response in smokers with asthma to a short-term course of oral corticosteroids has also been examined. An early study found that current smoking predicted an impaired FEV_1 response to oral corticosteroids in patients with unstable asthma (22). In a subsequent randomised, placebo-controlled crossover trial, 14 smokers, 10 ex-smokers and 26 never-smokers with asthma took oral prednisolone 40 mg daily or placebo for two weeks (23). This study demonstrated that pre-bronchodilator FEV_1, morning PEF and asthma control score improved in the never-smokers. However no change was observed in the smokers with asthma. A larger study examining corticosteroid response in smokers and non smokers with asthma found no difference in FEV_1 response when smokers and non smokers with asthma responses were compared. However non smokers with asthma demonstrated a larger PEF response than smokers with asthma (24).

An important diagnostic issue for the studies discussed above is whether the smokers recruited have asthma rather than chronic obstructive pulmonary disease (COPD) given the widespread recognition that subjects with COPD have a reduced response to corticosteroids (26). There can of course be overlap between the two conditions with some subjects displaying clinical features of both asthma and COPD. However the smokers recruited to the discussed studies had features that were more in keeping with asthma rather than a COPD phenotype. For example, the average onset of asthmatic symptoms were either early twenties (7, 9, 23) or mid thirties (8, 25). Patients were also required to demonstrate either airway hyperreactivity (7, 9) or reversible airflow obstruction following inhaled salbutamol of $\geq$12% (9) or $\geq$15% FEV_1 (23, 25) as a

requirement for entry to the trial helping to reduce the number of participants with COPD.

1.3 Corticosteroids-Role in the treatment of asthma and mechanisms of action

1.3.1 Corticosteroids-Discovery and therapeutic role

Following the discovery of the chemical structure and effects of corticosteroids synthetic analogues were rapidly developed for clinical use. As a result of their ability to control previously refractory inflammation, corticosteroid preparations became crucial to the management of patients suffering from debilitating inflammatory diseases including asthma. However the side effects associated with the prolonged use of oral preparations led to a rapid reduction in their prescription. Inhaled versions were subsequently developed for the treatment of asthma to reduce systemic exposure and alternative disease modifying medications have superseded corticosteroids in the management of rheumatoid arthritis and inflammatory bowel disease.

Since their introduction inhaled corticosteroids have become established as the cornerstone of asthma management due to their consistent ability to reduce symptoms, asthma exacerbations, improve lung function, suppress non-invasive markers of airway inflammation, eosinophil numbers, inflammatory cell activation and inflammatory gene transcription in the majority of patients (2, 27).

1.3.2 Corticosteroid resistance

However there are a significant minority of patients with asthma (and other inflammatory conditions) who fail to demonstrate the expected improvements in response to corticosteroids. Corticosteroids have been estimated to fail to control symptoms in 5-10% of non smoking asthmatics and this group is thought to be responsible for 50% of the total costs of asthma due to their increased symptoms and frequent admissions to medical wards and intensive care units (28, 29). However if also we include data from a recent asthma management

trial, total control of asthma by inhaled corticosteroids alone can only be achieved in 40% of non-smoking subjects with asthma (25).

Smokers with asthma are traditionally excluded from studies examining the efficacy of new treatments because of concerns about recruitment of subjects with chronic obstructive pulmonary disease. As a result little was known about their treatment response to corticosteroids until recently. However it is now clear that smokers with asthma display reduced therapeutic responses to corticosteroids.

1.3.3 Corticosteroids-Mechanisms of action

The activities of glucocorticoids can be divided into genomic and non-genomic effects based on the interactions with and through the glucocorticoid receptor with genomic being mediated via the glucocorticoid receptor (GR) and non genomic via both the GR and the cell membrane (30, 31).

The GR consists of three domains with differing functions. These are an N terminal domain, which carries out transactivation functions, a DNA binding domain and a ligand-binding domain. This receptor structure is common to the nuclear hormone receptor family which also contains the receptors for vitamin D, thyroxine, peroxisome proliferator-activated receptor- γ (PPARγ) and retinoic acid. However the GR has some unique features. It has a unique dimerisation domain, two charge clamps instead of one (unlike the other nuclear hormone receptors) and a distinct agonist binding pocket (32).

The gene for the GR is localised on chromosome 5 and consists of nine exons. Due to alternative splicing from these exons the GR has several isoforms (32, 33). The ubiquitously expressed and best studied form is glucocorticoid receptor alpha (GR-α). The GR-α is a 95 kDa protein composed of 777 amino acids. There is also heterogeneity within the GR-α as a number of variants are produced through differences in ribosomal translation. These variants have different ligand affinities and post ligand binding behaviour providing a mechanism for differing tissue corticosteroid sensitivities and between subject variation in corticosteroid response (33).

1.3.3.1 Cellular localisation and chaperones

Unbound, the GR resides in the cytoplasm, associated with a number of chaperone proteins including heat shock proteins 40, 56, 70, 90, the immunophilins p23 and Src and members of the mitogen associated protein kinase (MAPK) family (30, 32). Upon steroid binding the GR dissociates from these proteins, allowing formation of a homodimer and active nuclear translocation by importins due to exposure of its nuclear localisation sequence.

1.3.3.2 Transactivation

Glucocorticoid receptor homodimers interact with DNA response elements upstream of genes and recruit transcriptional co-activator proteins, which enhance transcription. This process is part of what is commonly referred to as transactivation. The DNA response elements contain palindromic repeats that facilitate glucocorticoid receptor binding and once bound the glucocorticoid receptor can increase and decrease gene expression (27, 30-32, 34). Through transactivation glucocorticoids can increase the production of a number of genes such as the NFκB chaperone protein IκB, dual specificity mitogen associated protein kinase phosphatase-1, glucocorticoid inducible leucine zipper, lipocortin/annexin-1, the cytokine IL-10, and surfactant protein D (32) and can also down regulate genes via negative gene regulation elements. Transactivation can also involve displacing transcription factors from response elements for inflammatory genes on DNA and occupying the vacated area (30, 32).

1.3.3.3 Transrepression

The glucocorticoid-GR complex can suppress inflammatory gene expression as a monomer through transrepression. This mechanism is postulated to be the main method by which corticosteroids suppress inflammatory gene expression (32). Transrepression can be performed through:

- *Direct inhibition*. Direct physical interaction between the GR and inflammatory transcription factors such as NFκB and AP1.

- *Chaperone proteins.* GR can stimulate increased production of chaperone proteins for inflammatory transcription factors such as the NFκB chaperone IκB.

- *Co-factor competition.* Competition for transcription co-factors required by both the GR and inflammatory transcription factors can reduce inflammatory gene expression.

- *Epigenetic mechanisms.* The GR can alter the behaviour of inflammatory transcription elements through actively recruiting multiprotein complexes capable of adding or removing covalent compounds such as acetyl and phosphate groups. This activity alters their affinity for their response elements and hence their behaviour (27, 30-32, 34).

1.3.3.4 Epigenetic effects

The field of epigenetics examines the effect of post-translational modifications of chromatin and associated proteins on the control of gene expression. This group of processes appear to be an important transrepressive mechanism exploited by corticosteroids.

The transcription machinery protein, RNA polymerase II, is unable to access promoter regions of DNA within chromatin's tertiary structures in chromatin's resting state due to DNA being tightly complexed with histone proteins. Histones consist of globular bodies with peripheral mobile tails and an overall positive charge which facilitates chromatin compaction. The addition of small molecules to histone tails such as acetyl or methyl groups and other modifications such as phosphorylation, ADP–ribosylation and SUMOylation have been demonstrated to affect transcription. Acetylation of histones has been recognised to be associated with increased gene expression for many years (35) and research using an *in-vitro* model has led to the estimate that approximately half of the immunosuppressant activity of corticosteroids is mediated through the removal of acetyl groups from histone tails (36).

1.3.3.5 Post translational modifications of glucocorticoid receptor

The GR is also subject to epigenetic modifications with resultant alteration in its behaviour. For example GR phosphorylation status affects its corticosteroid affinity, nuclear sub localisation and transactivation potential. Acetylation of the GR is also an important control mechanism as it affects its ability to interact with NFκB (32, 37). This raises the importance of post translational modifications of the GR by the mitogen associated protein kinase family and nuclear co-activator complexes to the response to corticosteroids.

1.3.3.6 Non-genomic effects of glucocorticoids

The non-genomic mechanisms by which glucocorticoids act are much more rapid than the genomic mechanisms but can still be inhibited by glucocorticoid receptor antagonists. These include effects produced by high concentrations of glucocorticoids where glucocorticoids are incorporated into both cell and mitochondrial membranes altering their properties. This results in a reduction in calcium and sodium ion transmembrane flux and mitochondria membrane ATP leakage (30).

Other non-genomic effects may be mediated through GRs imbedded in the cell membrane and through rapid changes in cytoplasmic concentrations of unbound GR chaperone proteins (30, 32). High concentrations of glucocorticoids also appear to be able to alter mRNA stability resulting in increased degradation of inflammatory gene mRNA and reduced inflammatory protein complex production (32).

1.3.3.7 Heterodimer formation

So far I have only considered homodimerisation and undimerised glucocorticoid receptors. However nuclear hormone receptors are capable of forming heterodimers with other transcription factors and the glucocorticoid receptor is no exception.

Recent work has identified functional interactions between the GR and the signal transducers and activator of transcription (STAT) and Ets families (32) and

with another nuclear hormone receptor, peroxisome proliferator activated receptor-γ (PPARγ)(38). For example GRα association with STAT5, a member of the STAT family, results in increased expression of β casein (32) and co-stimulation of cells with dexamethasone and a PPARγ agonist results in enhanced suppression of inflammation (38). The GRα can also form heterodimers with other members of the GR family such as GRß leading to altered behaviour (32).

1.4 Potential mechanisms of reduced corticosteroid sensitivity in smokers with asthma

Many defects in the complex action of corticosteroids have been described in a variety of clinical settings, but few have been directly attributed to cigarette smoking (32, 39, 40). However previous research examining corticosteroid resistance in non smokers with severe asthma & subjects with COPD coupled to *in-vitro* research examining the corticosteroid pathway and research utilising induced sputum in smokers with asthma can provide insights and evidence of possible causes.

1.4.1 Clinical studies-Sputum differential and supernatant

Smokers with asthma have been demonstrated to have altered sputum differentials and supernatant cytokine profiles compared to non smokers with asthma. One study comparing smokers and non smokers with asthma found that smokers with asthma displayed increased sputum cellularity, sputum neutrophils and reduced eosinophils (41). Smokers with asthma were also found to have increased sputum interleukin 8 (IL-8) levels. Sputum IL-8 was also found to positively correlate with sputum neutrophilia and pack years and to negatively correlate with lung function (FEV_1 pre bronchodilator). Another study demonstrated that sputum IL-18 expression and transcription is reduced in smokers with asthma and normal smokers compared to non-smokers with asthma and normal non-smokers (42). The smokers with asthma recruited to this study also had increased sputum neutrophils and reduced sputum eosinophils compared to non smoking asthmatics. The reduction in IL-18 was thought to reflect an alteration of the inflammatory profile in smokers with asthma as IL-18 has the ability to promote both Th1 and Th2 inflammation. Smokers with

asthma may therefore have a different Th1/Th2 profile to non smokers with asthma.

However smokers with asthma do not consistently display higher sputum neutrophils compared to non-smokers with asthma. A recent study found equivalent levels of sputum neutrophils in smokers and non smokers with asthma and in the same study sputum eosinophils were similarly suppressed in both groups (24). However this lack of a difference in sputum neutrophils may have been due to the two groups having more severe asthma and therefore more neutrophilia in the non smoking asthma group compared to the previous studies. The finding of equivalent levels of sputum eosinophils in this study can be explained by the increased use of inhaled corticosteroids in asthma compared to the preceding studies and hence suppression of sputum eosinophilia in the non-smokers with asthma.

The presence of sputum neutrophilia in some smokers with asthma is of interest given the association of airway neutrophilia with non smoking severe asthma. This group demonstrate relative corticosteroid resistance and worse asthma control (43-45). However not all smokers with asthma display sputum neutrophilia and therefore the presence of neutrophilia in a subgroup of smokers with asthma cannot explain all of the corticosteroid insensitivity displayed by this group.

Should smokers with asthma therefore be regarded to be part of what has been termed non-eosinophilic asthma? Non-eosinophilic asthma has been suggested to be comprised of two subgroups; a neutrophilic subgroup based on a cut off of sputum neutrophils >61% combined with poor asthma control and a paucicellular group with a normal sputum profile and good control (46). Smokers with asthma do not easily fit either of these subgroups given that neutrophilia is not present in all in this group and their level of asthma control cannot be described as good. This suggests that the alteration in airway cell proportions observed in smokers with asthma should be viewed as indicative of an alternative inflammatory response.

1.4.2 Clinical studies-Exhaled markers of inflammation

Exhaled nitric oxide (NO) has generated considerable interest due to its potential as a non-invasive marker of airway inflammation. Previous research suggests that exhaled NO may have a role in the monitoring of asthma due to correlation between exhaled NO levels and airway eosinophilia (47, 48). Exhaled NO levels have been assessed in smokers with asthma with comparison to non smokers with asthma (24, 49). A consistent finding is that smokers with asthma have an exhaled NO concentration level that is lower than non-smokers with asthma and equivalent to (or lower than) that observed in normal subjects. Exhaled NO concentrations also demonstrate a strong reciprocal correlation with pack year histories (50). The mechanism that produces this reduction in exhaled NO is not fully understood but may be due to increased consumption of NO (51, 52), competition for substrate from other inflammatory pathways (53) or reduction in production by inducible NO synthase (52, 54, 55). The consistent reduction of exhaled NO in smokers with asthma again suggests that a different form of inflammation is present in this group.

Exhaled breath condensate (EBC) is currently being examined as a potential non-invasive measurement of airway lining fluid (56). Studies utilising this method have principally focused on non smokers with asthma. A potentially important observation is that subjects with unstable asthma have reduced EBC pH which normalises with clinical improvement (57). One study has examined EBC pH in smokers and non-smokers with asthma and demonstrated a reduced EBC pH in smokers with asthma (58).

1.4.3 Clinical studies-Bronchoscopic samples

The airway wall has recently returned as a focus of attention in asthma due to the hypothesis that epithelial damage may be driving the inflammatory response (59) and the recognition that differing patterns of inflammation and structural responses are present in bronchial biopsies from different asthma sub types (60). A recent study compared bronchial biopsies from smokers with mild asthma with low pack year histories (mean (SEM) 16.7 (+/- 2.2)) against never smokers with mild asthma and found increased squamous metaplasia of the airway epithelium,

subepithelial neutrophils, subepithelial neutrophil elastase, intraepithelial IL-8 and interferon-γ mRNA expression in the smokers relative to non smokers (61). The authors concluded that their group of smokers with asthma displayed early evidence of a corticosteroid resistant phenotype similar to COPD and severe asthma.

Another bronchial biopsy study from the same group examined nitric oxide production in a group of smokers with asthma with low pack year histories (mean (SEM), 16.7 (+/- 2.2))(53). The amino acid L-arginine is the substrate for both the nitric oxide synthases and arginase-1 and hence is a point for substrate competition between the nitric oxide and ornithine pathways. In this small study a clear difference was evident in the expression of arginase 1 and ornithine de-carboxylase, with increased levels of both in smokers with asthma. Smokers and non smokers with asthma were found to have equal levels of the inducible nitric oxide enzyme iNOS. This finding supports the previous finding of reduced exhaled nitric oxide concentrations in smokers with asthma and suggests that smoking could be associated with a deviation from production of nitric oxide to proline and polyamines and hence increased airway remodelling.

A recent study which examined bronchial biopsies in smokers and non smokers with asthma considered the possibility that altered dendritic cell numbers and/or behaviour could contribute to the altered phenotype displayed by smokers with asthma (62). Smokers with asthma displayed lower levels of CD83 positive (a marker of mature dendritic cells) and CD20 positive cells (a marker for B lymphocytes) and preservation of Langerhans' cell numbers. The authors concluded that the altered dendritic cell and B cell phenotypes were the result of a different maturation state or the result of migration of both cells out the respiratory tract and that this alteration could be partly responsible for the altered response to corticosteroids in smokers with asthma.

A recently published study has examined the effect of smoking on alveolar macrophage inflammatory responses in asthma (63). Smokers with asthma displayed greater concentrations of macrophages in BAL fluid and BAL macrophages from smokers with asthma displayed a reduced cytokine response to LPS. This manifested as smaller increases in IL-6, 8 and TNFα in response to LPS compared to non-smoking asthmatics. However no difference was evident in

alveolar macrophage corticosteroid response. No baseline comparisons in bronchoalveolar cytokine levels were reported from this cohort. The authors suggested that this muted response to LPS may be indicative of an increased susceptibility to bacterial infection in smokers with asthma.

1.5 Alteration of the glucocorticosteroid pathway

Could the altered response to corticosteroids displayed by smokers with asthma be a result of alteration in the glucocorticoid pathway or in processes affected by this pathway? Previous research examining the corticosteroid pathways *in-vitro* and corticosteroid responsiveness in non smokers with severe asthma and subjects with COPD may provide insights for the investigation and treatment of smokers with asthma.

1.5.1 Glucocorticoid receptor

1.5.1.1 Cytokine induced corticosteroid resistance

Previous research examining corticosteroid resistance in non-smokers with asthma has identified associated alterations in pulmonary cytokine environments (64, 65). Could altered levels of pulmonary cytokines induce corticosteroid resistance or are they reflective of the reduced effect of corticosteroids? A partial answer has been provided through the use of the combination of IL-2 and 4 to induce corticosteroid resistance *in-vitro*. Kam and colleagues (66) demonstrated a reversible reduction in glucocorticoid receptor affinity in peripheral blood T-lymphocytes in subjects with corticosteroid resistant asthma. T lymphocytes from the recruited subjects had reduced glucocorticoid receptor affinity which recovered after a few days in culture. However on addition of a combination of IL-2 & 4 this binding defect was restored. This finding has been replicated a number of times (67-69) and is reversible following the addition of IFNγ (66, 69).

The mechanisms by which the combination of IL-2 and 4 could induce altered glucocorticoid receptor behaviour have been examined in a series of experiments (68, 69). One group used peripheral blood mononuclear cells (PBMC) pre-treated with IL-2 and IL-4 and subsequent exposure to lipopolysaccharide to

demonstrate that the defect in ligand binding to the glucocorticoid receptor was due to activation of the p38 MAPK pathway and phosphorylation of the glucocorticoid receptor (68). This work has subsequently been confirmed and appears to be reversed by concomitant administration of IFNγ (69). Another study examined LPS stimulated cytokine responses in alveolar macrophages obtained by bronchoalveolar lavage from non-smoking subjects with severe and mild asthma and normal volunteers (70). Alveolar macrophages from subjects with severe asthma demonstrated a reduced anti-inflammatory response to dexamethasone with associated increased p38 MAPK activation. GRα is a target for phosphorylation by both JNK and p38 MAP kinase, and activation of these pathways leads to a reduced corticosteroid response (68-71). Therefore cigarette smoke induced alteration in the pulmonary cytokine environment may be capable of altering corticosteroid responses through activation of MAPK pathways.

1.5.1.2 Alternative glucocorticoid receptor isoforms

The corticosteroid receptor exists in several forms, with the best studied being the alpha (GRα) and beta (GRß). The GRα is responsible for the therapeutic effects of corticosteroids. In contrast GRß can interfere with GRα function in a dominant negative fashion. This has led to their being termed decoy receptors and research has demonstrated a potential role in steroid resistant asthma for GRß with a reduced ratio of α: ß and increased ß-receptor levels being linked to corticosteroid resistance (72-76).

A small sub-study has provided evidence for cigarette smoke exposure producing a reduction in both GRα and ß expression in normal smokers and subjects with COPD (77). Research examining the α:ß receptor ratio in peripheral blood mononuclear cells from smokers with asthma has also demonstrated a reduced α:ß ratio suggesting that an increase in the ß form and/or a decrease in the α form may have a role in corticosteroid resistance in smokers with asthma (78).

1.5.1.3 Altered glucocorticoid receptor kinetics

Glucocorticoid receptor density and ligand binding characteristics have been examined in peripheral blood mononuclear cells (PBMCs) from non smoking

subjects with severe asthma (79, 80). In a small study of subjects with corticosteroid sensitive and resistant asthma PBMC GR binding affinity and GR number was found to be equivalent (79). This finding has been corroborated in a slightly larger study comparing subjects with mild and severe asthma (80). However evidence does exist for differences in GR binding in severe asthma. PBMC nuclear extracts from subjects with severe asthma demonstrated reduced GR binding affinity in one trial (68) and in another study reduced binding affinity in peripheral blood T lymphocytes was present in some subjects with severe asthma with evidence for reduced GR concentrations in a small subgroup (67).

Therefore it is feasible that smokers with asthma may display altered GR binding behaviour, receptor number, an increase in the β or reduction in the α subtype or post translational modifications compared to non-smokers with asthma. Future work should examine the GR in smokers with asthma in light of this work.

1.6 Acquired defects in HDAC activity and potential implications for corticosteroid responses

Corticosteroids exert their effects on gene expression via many mechanisms and pathways. One important mechanism is the control of epigenetic changes. Epigenetics involves the addition of small molecules such as acetyl or methyl groups and other modifications such as phosphorylation, ADP-ribosylation and SUMOylation (SUMOylation = reversible conjugation of a <u>s</u>mall <u>u</u>biquitin-related <u>mo</u>difier protein to another protein) to histone tails and other proteins and the effect that this has on transcription. I shall now briefly examine how control of protein acetylation by corticosteroids through manipulation of HDACs and histone acetyltransferases (HAT) could affect chromatin structure and gene expression and how alteration of these mechanisms could be responsible for the altered response to corticosteroids in smokers with asthma.

Several enzymes have been identified to have HDAC activity. 18 HDAC isoforms are currently recognised and are divided into three groupings, class I & II and the sirtuins. HDACs form part of several important intracellular multiprotein complexes that are involved in transcription control and are expressed throughout the lung, with the highest levels found in airway epithelial cells and

alveolar macrophages (81). A proposed mechanism for the reduced response to corticosteroids in COPD and steroid resistant asthma is that oxidative stress, either from active smoking or other sources, reduces HDAC activity impairing the ability of corticosteroids to reduce inflammation (27, 82). No published work is available from studies in smoking asthmatic patients examining HDAC activity, but inferences can be drawn from *in-vitro* studies and from several studies in non smokers with asthma, subjects with COPD and normal smokers.

1.6.1 HDAC activity and expression in asthma

Bronchial biopsies from atopic non smokers with mild asthma (treated with β-agonists alone) and mild to moderate non-smoking asthmatics (treated with inhaled corticosteroids) were compared to a group of normal non-smoking volunteers in one study (83). The intensity of staining for the isoforms HDAC1 & 2 was reduced in asthmatics compared to normal subjects. However subjects with asthma treated with inhaled corticosteroids demonstrated a restoration of total HDAC activity and HDAC2 expression and reduction in HAT activity towards normal levels. In a subsequent study, which examined acetylation status in airway macrophages, HDAC1 expression was found to be reduced in mild asthmatics with no alteration in HDAC2 or 3 expression (84). Another study from the same group examined PBMC cytokine responses in subjects with severe asthma, mild asthma and normal subjects and identified a reduction in HDAC activity in subjects with severe asthma which was associated with a reduced anti-inflammatory effect of dexamethasone *in-vitro* (85).

1.6.2 HDAC in COPD

Two trials have examined acetylation balance in COPD (86, 87). The first study (86) examined both HDAC and HAT activity and HDAC isoform expression levels in peripheral lung tissue from subjects with COPD of various degrees of severity. When compared with normal subjects total HDAC activity was reduced in subjects with severe COPD. This reduction was found to positively correlate with FEV_1 and was associated with a reduction in expression of HDACs 2, 5 and 8. No difference was found in the level of expression of HDACs 1, 3, 4, 6 and 7. Similar

results were obtained using bronchial biopsies and macrophages obtained by bronchoalveolar lavage.

Another study examined subjects with milder COPD using tissue obtained from lung resections (87). Smoking played a critical role in acetylation status. Smokers with and without COPD were found to have a doubling in acetylated histone-4 levels compared to normal non-smokers suggesting a reduction in HDAC activity. In contrast ex-smokers with COPD demonstrated a return to normal non-smokers' histone acetylation levels. This reduction was not global however as the ex-smokers with COPD were found to have a quadrupling of acetylated histone-3 levels compared to normal non-smokers. In contrast to the first study (86), HDAC2 nuclear expression was equivalent. However this may reflect the milder phenotypes recruited.

1.6.3 Oxidative stress, smoking and HDAC

How cigarette smoke alters HDAC activity and gene expression is not fully understood but recent research has identified a role for increased oxidative stress due to cigarette smoking. Cigarette smoke contains at least 10^{15} free radicals per inhalation in a mixture of short lived species such as superoxide and longer living compounds such as tar-semiquinone (88). Each substance is capable of altering airway cell composition and intracellular signalling (89) and oxidative stress appears to play a crucial role in the development of COPD (90, 91).

Experimentally induced oxidative stress is able to reduce HDAC activity to the levels seen in COPD and asthma. For example, hydrogen peroxide (H_2O_2) added to cell lines reduces HDAC expression and dexamethasone mediated suppression of cytokines to a level seen in smokers (92). Cigarette smoke condensate and H_2O_2 can both increase histone 4 acetylation and HAT activity and reduce HDAC2 expression and HDAC activity (93). Exposure of rats to cigarette smoke also reduces HDAC2 activity with associated increased histone acetylation and corticosteroid resistant inflammatory gene expression (94).

The reduction in HDAC activity following oxidative stress may be due to post-translational covalent modification of HDAC enzymes. HDAC2 exposed to cigarette smoke contains increased 4-hydroxynonanol (4HNE) and nitrated

tyrosine motifs compared to controls (93, 94). Similar findings have been found when H_2O_2 was used as the oxidative stimulus (95). The relevance of cigarette smoke induced IL-8 production and its relationship to HDAC activity in COPD has been also examined *in-vitro* (96). IL-8 production was found to be increased on exposure to cigarette smoke with associated reductions in HDAC1, 2 and 3 expression and HDAC activity. Cigarette smoke also altered HDAC1, 2 and 3 as they demonstrated increased immunoreactivity for 4HNE and nitrotyrosine antibodies. The mechanism by which this modification reduces HDAC activity is thought to be via the addition of 4HNE to histidines within the HDAC active site and/or destruction of HDACs via the ubiquitin-proteasome pathway.

A recent publication examined the effect of cigarette smoke extract on HDAC2 in cell lines and a mouse model, and provided evidence for a role for HDAC phosphorylation and subsequent destruction via the ubiquitin-proteasome pathway in oxidative stress induced reduction in HDAC activity (97). Exposure of cells to cigarette smoke extract resulted in a decrease in expression of HDAC1 and 2 by four hours. Cigarette smoke extract was also demonstrated to cause rapid and transient phosphorylation of HDAC2 which peaked within half an hour of exposure and was reversed by two hours. The reduction in phosphorylation was followed by ubiquitination and evidence of destruction of HDAC2 via the ubiquitination-proteasome pathway.

1.6.4 Corticosteroids and HDAC

What are the cellular pathways that link glucocorticoids, the glucocorticoid receptor, HATs and HDACs? One group has examined the effect of dexamethasone on a model of inflammation and the relevance of these mechanisms (98). IL-1ß induced GM-CSF expression and response to the application of dexamethasone was assessed in an epithelial cell line. Dexamethasone reduced IL-1ß mediated acetylation of the tail of histone 4 lysine residues 8 and 12 and GM-CSF expression. This effect on GM-CSF production required dexamethasone binding to the glucocorticoid receptor and increased HDAC activity. Half of the reduction in GM-CSF was due to a dexamethasone induced increase in HDAC activity as demonstrated through the application of an HDAC inhibitor. Further examination identified increased

binding of the HDAC2 isoform to the NF-κB subunit p65 following dexamethasone suggesting increased recruitment of HDAC2 to NF-κB was responsible for the inhibitory effect of dexamethasone at low doses. Therefore at low doses of corticosteroids the glucocorticoid receptor is able to carry out transrepression of gene expression through stimulating the interaction of HDAC2 with the p65 subunit of NF-κB.

Subsequent work, utilising the same model, which compared the effect of the dissociated steroid RU486 (mifepristone) and dexamethasone confirmed this observation (36). HDAC2 activity is crucial for this corticosteroid mediated inhibition of inflammation as demonstrated by graded reduction of HDAC2 with siRNA (36, 37). HDAC2 knockdown did not affect either GR nuclear translocation, GR-GRE binding, glucocorticoid induced gene expression or the ability of high dose dexamethasone to inhibit GM-CSF production. However following HDAC2 knockdown the GR is not recruited to the NF-κB-DNA complex and therefore is unable to inhibit NF-κB activity.

The glucocorticoid receptor is also subject to acetylation with effects on its transrepressive activity (37). When interacting with and inhibiting the NF-κB-DNA complex the glucocorticoid receptor is normally not acetylated. However following acetylation of the receptor a strongly correlated reduction in dexamethasone mediated inhibition of NF-κB is observed. Airway macrophages obtained by bronchoalveolar lavage from patients with COPD were also found to have increased levels of glucocorticoid receptor acetylation and reduced HDAC2 expression. HDAC2 expression levels were then restored through vector induced HDAC2 over-expression resulting in reduced GR acetylation and restoration of suppression of GM-CSF expression.

1.7 Theophylline, HDAC and corticosteroids

Based on the existing clinical research it is clear that inhaled corticosteroids, the best therapy for asthma, are less effective than would be desired in smokers with asthma. This is certainly true for their short term ability to improve lung function, reduce symptoms and exacerbations. The most obvious clinical approach to the management of smokers with asthma is to encourage smoking

cessation. Smoking cessation is useful in this group as it has been demonstrated to produce large improvements in lung function (49). Unfortunately successful quitters take many years to achieve complete cessation (99). Clearly demanding smoking cessation alone for smokers with asthma is therefore an unacceptable approach for management of this group.

An alternative approach would be to investigate the effect of high dose inhaled corticosteroids in smokers with asthma based on previous research (8, 10). However this is unlikely to be a successful approach given the increased side effects and reduced compliance that would occur as a result. Therefore the search should begin for alternative effective treatments for this group. Again we can extrapolate from previous research examining alternative treatments in non smoking subjects with severe asthma, subjects with COPD and from *in-vitro* research and animal models of asthma.

The oral bronchodilator theophylline has been utilised in asthma and COPD for many years and is currently advocated as an add-on therapy in those subjects not controlled on inhaled steroids +/- LABA (1). Theophylline appears to have a number of mechanisms of action (100). It produces its bronchodilator effects through blockade of phosphodiesterase (PDE) activity, specifically PDE isoforms 3, 4 & 5, resulting in an increase in intracellular cAMP and smooth muscle relaxation. However significant PDE inhibition is unlikely to be achieved clinically due to the large doses that would be required and it has been suggested that at recommended doses theophylline is producing only about 5-10% of its maximum possible PDE inhibition (100). Theophylline has also demonstrated activity as an adenosine receptor antagonist and can block A1 and A2 receptors producing relaxation of airway smooth muscle and stabilising mast cells as a result. However the relative contribution of this activity to control of bronchospasm and inflammation is currently unclear. Another therapeutic effect that has been demonstrated for theophylline is an ability to increase HDAC activity at low serum concentrations (around 3-7mg/dl, normal range 10-20mg/dl).

1.7.1 Theophylline increases HDAC activity & potentiates corticosteroid mediated suppression of inflammation

In a double blind crossover trial incorporating bronchial biopsies, 14 mild stable asthmatics treated with ß-agonist alone were treated with low dose theophylline for one month (101, 102). Treatment with theophylline was associated with increased HDAC activity in bronchial biopsies. This increase in HDAC activity was due to increased HDAC1 expression as HDAC2 was unaltered. Theophylline serum levels were low with a mean concentration of 6.1 mg/dl.

Subsequent to this trial a model of inflammation based on stimulating BAL macrophages with LPS was utilised to examine interactions between low dose theophylline and corticosteroids (102). After exposure to LPS, BAL macrophages displayed reduced HDAC activity and increased IL-8 production which was insensitive to 10^{-10}M dexamethasone. Dexamethasone at 10^{-6}M did succeed in restoring HDAC activity to normal levels and this was associated with a 60% reduction in the level of IL-8 production. Theophylline alone at a dose of 10^{-5} M was able to restore HDAC activity to normal without reducing IL-8. At higher concentrations the ability of theophylline to boost HDAC activity was lost. Theophylline (10^{-5} M) was then combined with low dose corticosteroid (10^{-10}M) resulting in increased HDAC activity and reduced IL-8. To further examine the ability of low dose theophylline to increase HDAC activity the HDAC inhibitor Trichostatin-A (TSA) was then applied. TSA reversed the suppression produced by the combination of 10^{-5} M theophylline and 10^{-10}M corticosteroid and reduced the suppressive ability of 10^{-6}M dexamethasone by 50%. Theophylline was found to increase the HDAC activity of HDAC1 and 3 but did not alter HDAC2.

A similar study has also been carried out using BAL macrophages from COPD patients, normal smokers and normal non-smokers (103). HDAC activity was significantly reduced in BAL macrophages from subjects with COPD and this correlated with the HDAC2 expression level. Low dose theophylline increased HDAC activity without altering IL-8 levels and low dose dexamethasone again reduced IL-8 when combined with theophylline. The IL-8 response to dexamethasone in normal smokers and subjects with COPD was reduced compared to normal non-smokers and was associated with increased NF-κB

nuclear translocation. The addition of theophylline to LPS-stimulated BAL macrophages from subjects with COPD potentiated the suppression of IL-8 produced by dexamethasone. Immunoprecipitated HDAC1 and 2 from normal smokers' BAL macrophages and a cell line were exposed to theophylline and in contrast to previous findings (102), both HDAC1 and 2 demonstrated increased HDAC activity on exposure to theophylline.

Low dose oral theophylline has also been examined in the recovery phase of COPD exacerbations (104). Subjects randomised to theophylline in addition to normal care (which included oral corticosteroids) demonstrated increased sputum macrophage HDAC activity and greater suppression of IL-8 and TNFα compared to subjects receiving standard care alone.

1.7.2 Mechanisms by which theophylline may increase HDAC activity

Several potential mechanisms by which theophylline could increase HDAC activity have been addressed in one study (102). Theophylline is known to cause bronchodilatation through non-specific inhibition of phosphodiesterases (PDE). The ability of theophylline to inhibit PDEs and the effect of this on HDAC activity was examined using non-specific and selective PDE inhibitors. The authors demonstrated that PDE inhibition was not contributing to theophylline's ability to increase HDAC activity. Previous research has also demonstrated that phosphorylation of HDAC isoforms can increase their HDAC activity (105-108). Therefore the effect of phosphatases on the ability of theophylline to increase HDAC activity were also examined using alkaline phosphatase, a p38 MAPK inhibitor (SB203580) and a MEK inhibitor (PD098059). Pre-treatment with alkaline phosphatase was found to reduce theophylline's ability to increase HDAC activity by about 40%, providing evidence for a role for phosphorylation in increasing HDAC activity. The p38 inhibitor SB203580 also reduced HDAC activity suggesting that theophylline utilises p38 activation to increase HDAC activity. However inhibiting p38 did not completely abolish theophylline's HDAC effect so another phosphorylation pathway or other mechanisms may be involved. No effect on HDAC activity was seen with MEK inhibition. The final potential mechanism examined in this study was the possibility that theophylline utilises

allosteric interactions with HDAC enzymes to increase their activity. Theophylline's improvement in HDAC activity ranged from a 40% increase at pH 8 to 75% at pH 7.8 suggesting that theophylline may also employ allosteric interactions in addition to HDAC phosphorylation to increase HDAC activity.

Oxidative stress may also play a role in the ability of theophylline to restore corticosteroid sensitivity. Work using cell lines has demonstrated that oxidative stress reveals intracellular targets for theophylline with associated restoration of corticosteroid sensitivity and reduction in histone acetylation at inflammatory genes (109). In the context of oxidative stress theophylline was also able to induce genes that counteract the effects of this stress and to suppress the expression of genes related to oxidative stress induced pathways.

Recent work has also identified that theophylline can act as a phosphatidylinositol 3 kinase (PI3K) inhibitor and it has been suggested that this ability may be responsible in part for theophylline's ability to increase HDAC activity (110-112). Investigation of the PI3Kδ isoform has been carried out in an animal model of cigarette smoking pulmonary inflammation (77). Work using this model demonstrated that PI3Kδ is involved in the development of reduced corticosteroid sensitivity following exposure to cigarette smoke. This was associated with a reduction in total HDAC activity and increased tyrosine nitration of HDAC2.

1.7.3 Possible role for theophylline in the treatment of smokers with asthma

The available research suggests that HDAC activity may be low in patients with COPD and asthma and that this can be corrected through the use of low dose theophylline. However this is likely to only have clinical benefits when subjects are treated with inhaled corticosteroids in combination with theophylline as increased HDAC activity alone does not appear to be sufficient to suppress inflammation. Smokers with asthma display a blunted response to inhaled and oral corticosteroids but the mechanism by which this occurs is unknown. Previous research has suggested that smokers with asthma may partially respond to high dose inhaled corticosteroids (8, 10). This response mirrors the results

discussed in the preceding section. It is tempting to speculate that the reduced response to inhaled and oral corticosteroids displayed by smokers with asthma is a result of cigarette smoke mediated inhibition of HDAC activity and that this could be restored by low dose theophylline. The studies discussed above suggest that the addition of low dose theophylline *in-vitro* can produce the same effect as increasing dexamethasone dose by 100-1,000 times. This effect is unlikely to be as marked *in-vivo* due to local mechanisms which exert control over corticosteroid concentrations. However examination of low dose theophylline in combination with low dose inhaled corticosteroids appears to be merited in smokers with asthma based on the available evidence.

1.8 PPARγ and inflammation

1.8.1 Is there a role for PPARγ agonists in smokers with asthma?

Given the failure of corticosteroids to produce their expected effects in smokers with asthma alternative therapies are required for this group. Could a new class of therapeutic agents directed at an alternative anti-inflammatory pathway reduce the cigarette smoking induced inflammation that is present in smokers with asthma? A large body of literature detailing the anti-inflammatory effects of peroxisome proliferator-activated receptor (PPARs) agonists in animal and *in-vitro* models of asthma has developed over the past decade and has led to an interest in the possible role for PPARγ stimulation in the treatment of asthma and other inflammatory conditions (113, 114).

1.8.2 PPARs-Discovery & structure

PPARs, like the glucocorticoid receptor and the receptors for thyroxine and vitamin D, belong to the nuclear hormone receptor superfamily and are ligand-inducible transcription factors. PPARs were first described following the observation that certain compounds (for example; fibrates, phthalate esters, non-steroidal anti-inflammatory drugs) could increase the number and activity of liver peroxisomes after chronic high dose administration to rodents (115, 116). Peroxisomes are intracellular organelles, which perform diverse metabolic

functions including β-oxidation of fatty acids and have a role in cholesterol metabolism.

PPAR possesses four structural domains. The A/B region is a ligand-independent transcriptional activation domain (also known as activation-function 1/AF-1). The C domain encodes the DNA binding domain that contains two zinc finger motifs. The D domain codes for a hinge which is thought to allow movement of the ligand-binding domain relative to the DNA binding domain. The E domain is responsible for ligand binding, dimerisation, nuclear translocation and association with activators and repressors of transcription through its transactivation domain (activation function 2/AF-2).

1.8.3 PPAR family

PPARs exist in three isoforms; PPARα, PPARß/δ and PPARγ. The PPARs differ in gene and chromosome origin, display varied effects and have different tissue distributions. However the known PPAR isoforms do display strong structural and sequence homology. PPARα is expressed in heart, liver, kidney, adipose tissue and skeletal muscle, PPARß/δ is widely expressed in tissues such as bowel, heart, muscle, lung and adipose tissue and PPARγ is found at highest concentrations in adipose tissue (115, 116). PPARγ is also expressed in the lung epithelium, submucosa and airway smooth muscle and expression appears to be upregulated in response to inflammation (117).

1.8.4 PPARγ-Endogenous ligands

Previous research has led to proposals for the reaction products of polyunsaturated fatty acids and eicosanoids to be recognised as the endogenous PPARγ ligands. Polyunsaturated fatty acids are metabolised to produce agonists such as α-linoleic, γ-linolenic, arachidonic and eicosapentaenoic acids. Eicosanoid metabolites produce the agonists 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$), 9-hydroxyoctadecanoic acid (9-HODE), and 13- hydroxyoctadecanoic acid (13-HODE). 15d-PGJ$_2$ is formed from PGD$_2$ and has been demonstrated to bind to PPARγ and has been proposed to be the principle endogenous PPARγ agonist (113, 118). However there is substantial evidence that 15d-PGJ$_2$ acts

through more than just PPARγ stimulation (119-124). Examples include experiments where saturating concentrations of synthetic PPARγ ligands fail to block the effects of 15d-PGJ$_2$ *in-vitro* (125) and different concentrations of 15d-PGJ$_2$ have been observed to exert differing effects (126). Therefore interpretation of results from experiments using 15d-PGJ$_2$ as a pure PPARγ agonist effect has been criticised (127).

1.8.5 PPARγ-Synthetic Ligands

Commonly prescribed drugs can stimulate PPARγ. These include non-steroidal anti-inflammatory drugs (NSAIDs), fibrates (128), retinoids (129) and the thiazolidinediones. Thiazolidinediones are potent PPARγ agonists and were designed to exploit the beneficial effect of PPARγ stimulation in the treatment of diabetes mellitus. The use of these compounds in PPARγ research has led to a better understanding of the role of PPARγ in metabolism and inflammation. Similar to 15d-PGJ$_2$ there has been some debate over whether the anti-inflammatory effects of thiazolidinediones are mediated exclusively via the PPARγ receptor (130-132). However an analogy can be drawn with glucocorticoids which can exert anti-inflammatory actions via and independent of the glucocorticoid receptor. Given the family homology there are some similarities in the mode of action of PPARγ and the glucocorticoid receptor and PPARγ can also reduce inflammation via transactivation and transrepression and via a number of non-genomic pathways

1.8.6 Anti inflammatory effects of PPARγ and thiazolidinediones

Following the discovery of PPARγ and the evidence for its involvement in resolution of inflammation many groups have investigated its role in disease models and patients. As a result PPARγ's role in a variety of inflammatory conditions such as atherosclerosis (133-135), inflammatory bowel disease (136, 137), acute lung injury (138) and pulmonary fibrosis (139) has come under investigation. A large body of evidence also exists for PPARγ agonists having anti-inflammatory effects in *in-vitro* and animal models of asthma and inflammatory airways disease.

Specific examples include:

- *Eosinophils*. Reduced eosinophilic inflammatory response in ovalbumin allergen challenge models (140-142).

- *Neutrophils*. Reduced neutrophilic response to LPS in an animal model designed to simulate neutrophilic ling disease (143) and reduced neutrophil chemotaxis (144).

- *T lymphocytes*. Inhibition of T lymphocyte clonal proliferation (119, 145-148) and induction of T lymphocyte apoptosis (149).

- *Dendritic cells*. Altered dendritic cell maturation and behaviour in response to stimulation (141, 150, 151).

- *Macrophages*. Altered macrophage maturation (119, 123, 128, 152-155), cytokine production (119, 123, 152, 156, 157) and evidence of PPARγ stimulation resulting in increased phagocytic potential for apoptotic neutrophils (154).

- *Airway Epithelial cells*. Increased expression of PPARγ in human asthma (117) and animal models (158) and reduction in airway hyperresponsiveness and mucus production, collagen deposition, basement membrane thickness and transforming growth factor-ß (TGFβ) synthesis (158) and reduced epithelial cell cytokine expression (159, 160).

- *Airway Smooth muscle*. Reduced smooth muscle cell proliferation (124) and cytokine expression (38, 161).

- *Airway Fibroblasts*. Inhibition of differentiation to myofibroblasts and cytokine production (162).

1.8.7 PPARγ modes of action

There are similarities between corticosteroids and PPARγ agonists in their ability to inhibit multiple inflammatory cells and their efficacy in animal models of

asthma. Similarities also exist in their multiple modes of action. PPARγ has been demonstrated to exert its anti-inflammatory effects using the following mechanisms.

1.8.7.1 Transactivation

The binding of an agonist to PPARγ induces a conformational change. This allows dissociation of co-repressor molecules and association with co-activators. PPARγ then forms heterodimers with retinoic X receptor (RXR) and binds to peroxisome proliferator response elements (PPREs) in DNA altering gene expression. As a result PPARγ exerts control over a wide number of overlapping but distinct genes from the glucocorticoid receptor (163).

1.8.7.2 Transrepression

PPARγ stimulation may produce its effects via inhibition of inflammatory gene transcription (transrepression) and there are a number of ways that this may occur (115, 164, 165):

- *Sequestration of shared co-activators*. PPARγ activation may reduce the supply of common co-activators reducing their availability to inflammatory transcription factors.

- *Control of IκB kinase (IKKB kinase)*. IKKB releases NF-κB from inhibition by phosphorylating its inhibitory protein IκB (which subsequently leads to its degradation and increased NF-κB activity)(120).

- *SUMOylation of the PPARγ ligand-binding domain*. SUMOylation of PPARγ facilitates PPARγ mediated transcriptional suppression of inflammatory genes (165, 166)

- *Inhibition of inflammatory transcription factors*. Examples exist for NF-κB (167), NFAT (128, 164, 168) & AP1 (146).

- *Upregulation of anti-inflammatory genes*. The tumour suppressor molecule PTEN (phosphate and tensin homologue deleted on chromosome

ten) is a phosphatase known to be downregulated in asthma. PPARγ stimulation increases its concentration in animal models of asthma with associated reduced inflammation (142).

1.8.7.3 Non genomic effects

- *MAPK stimulation*. PPARγ agonists exert some of their effects far too rapidly to be working via gene transcription or suppression, and recent evidence has suggested that they can produce some of these rapid effects via mitogen associated protein kinases (MAPK) (128, 157, 169-172).

- *Intracellular organelles*. PPARγ agonists exert some effects via intracellular organelles independent of PPARγ:

  - *Endoplasmic reticulum*. Ciglitazone and troglitazone have been demonstrated to increase intracellular calcium by directly stimulating its release from the endoplasmic reticulum (173).

  - *Mitochondria*. Other groups have demonstrated interaction between mitochondria and PPARγ agonists (132, 174, 175). Thiazolidinediones alter mitochondrial respiration but appear to have effects beyond this as illustrated by their ability to increase the heat shock response (132).

1.8.7.4 Modulation and utilisation of the glucocorticoid receptor by PPARγ

Two independent groups have demonstrated a functional interaction between PPARγ and the glucocorticoid receptor (GR). Nie et al (38) demonstrated direct physical interaction between PPARγ and the dominant glucocorticoid receptor, GRα, following 15d-PGJ$_2$ application in human airway smooth muscle cells. This interaction was functional as it resulted in the inhibition of eotaxin production by the cells following stimulation with TNF-α. Ialenti et al (176) have also examined PPARγ and GR interaction in a model of inflammation. Glucocorticoid receptor blockade with RU486 (a glucocorticoid receptor antagonist) removed a substantial portion of the anti-inflammatory effect of the PPARγ agonists rosiglitazone and ciglitazone. A PPARγ antagonist used in conjunction with RU486

was necessary to remove all the anti-inflammatory effects. In the same paper rosiglitazone and ciglitazone were observed to stimulate GR nuclear translocation in a PPARγ deficient cell line leading to the conclusion that PPARγ agonists could produce anti-inflammatory effects via GR activation. This work suggests that PPARγ agonists may be able to modulate existing glucocorticoid receptor activity to reduce inflammation and that combined therapy with PPARγ agonists and corticosteroids may produce a greater degree of control over inflammation than corticosteroids can achieve alone.

A recent paper has demonstrated evidence for direct stimulation of the glucocorticoid receptor by thiazolidinediones (177). In this study rosiglitazone was found to induce glucocorticoid receptor phosphorylation, nuclear translocation and increased expression of a glucocorticoid receptor dependent gene. The authors also demonstrated similar effects for other thiazolidinediones and suggested that thiazolidinediones may be exerting some of their anti-inflammatory and anti-diabetic actions through stimulation of the glucocorticoid receptor as a partial agonist.

1.8.7.5 PPARγ & HDAC

PPARγ appears to mediate part of its transrepressive actions via HDAC containing multiprotein complexes (165). A murine model of inflammation based on macrophage stimulation by lipopolysaccharide and production of iNOS demonstrated that the multiprotein complex NCoR, which contains HDAC3, suppresses transcription of a number of inflammatory genes in unstimulated cells by binding to gene promoters. Upon stimulation of the cell with LPS, NCoR was removed permitting NF-κB responsive inflammatory gene production. However addition of the PPARγ agonist rosiglitazone was found to result in SUMOylation of PPARγ on its ligand binding domain followed by PPARγ binding to the NCoR complex localised at the iNOS promoter. This prevented NCoR removal and transcription of NF-κB responsive inflammatory genes. This prolongation of transrepression was dependent on the HDAC activity of the NCoR complex, as rosiglitazone mediated transrepression was abolished through the use of the HDAC inhibitor Trichostatin A and siRNAs for HDAC3.

1.8.8 PPARγ & asthma

Few researchers have addressed the relevance of PPARγ to humans with asthma. One study has examined PPARγ expression in bronchial biopsies from corticosteroid naive asthmatic patients (117). The authors demonstrated that PPARγ expression was increased in corticosteroid naive asthmatic subjects compared to controls. This increased expression was evident in all epithelial compartments (epithelial cells, smooth muscle cells and bronchial mucosal cells) and PPARγ expression was reduced towards normal levels following treatment with inhaled corticosteroids.

What is the role played by PPARγ in asthma and what could be the reason behind its suppression following corticosteroid treatment? PPARγ is upregulated by IL-4, due to IL4 response elements within the PPARγ gene. A reciprocal relationship exists as PPARγ stimulation also inhibits IL-4 and this and similar work has led to a discussion over PPARγ having a role as an innate mechanism for resolution of inflammation (128). PPARγ stimulation also suppresses a number of other inflammatory cytokines of relevance to asthma and its upregulation by cytokines would support a role in the resolution of inflammation.

Variations in PPARγ also appear to be relevant to asthma control. A recent study examined single nucleotide polymorphisms (SNPs) in PPARγ in a large cohort of young subjects with asthma (178). Three previously characterised haplotypes were examined, Pro12Ala, C1431T & C-681G, using buccal cells. The subjects homozygous for the Pro12 and C1431 SNPs had increased asthma exacerbations and the combination of Pro12 and C1431 was associated with increased school absences and hospital admissions. Subjects homozygous for the Ala12 and T1431 SNPs in contrast had better asthma control. In summary the human research so far supports the animal and *in-vitro* data suggesting that PPARγ may have a role in the control of inflammation in asthma.

1.8.9 PPARγ and smokers with asthma

A central theme to this thesis is that smokers with asthma fail to gain the expected benefits from inhaled corticosteroids and require new therapies. The

presented evidence for PPARγ suggests that it has a role to play in the control of inflammation and as such may offer an alternative approach for the treatment of smokers with asthma. PPARγ expression is upregulated in asthma and reduces in response to corticosteroids. It is tempting to speculate that PPARγ expression is upregulated in smokers with asthma in correlation with their reduced corticosteroid response and as a result PPARγ stimulation could provide an alternative method for the reduction of inflammation in this group. Whilst evidence in support of this particular hypothesis is not available for smokers with asthma there is sufficient evidence to justify examination of a PPARγ agonist in smokers with asthma in an exploratory clinical trial.

1.9 Non-invasive assessment of inflammation

1.9.1 Rationale for use of non-invasive methods

Previous research investigating asthma has utilised autopsy tissue from cases of fatal asthma and samples obtained by bronchoscopic biopsies. Whilst autopsy samples are obviously not reflective of normal asthma, bronchoscopy does allow sampling of airway cells and bronchial tissue from a variety of subjects over a number of timepoints. Unfortunately bronchoscopy is associated with a degree of risk, albeit small in most, but significant in those with poor lung function. Bronchoscopy is also expensive both for the research unit due to the number of staff required to perform the test and for the patient given the amount of time required to recover. Therefore whilst bronchoscopy provides useful insights into asthma, concern over the high cost and validity of extrapolation to other subjects has resulted in the development of a number of non-invasive measures for the assessment and phenotyping of asthma and to follow treatment responses.

1.9.2 Induced sputum

The examination of patient's sputum for infective organisms is a well established diagnostic tool. The examination of sputum from asthmatics with respect to the cellular profile led to several important early observations including the association between sputum eosinophilia and good corticosteroid response (179).

However the use of spontaneous sputum is not ideal. Not all patients can produce sputum spontaneously and even when possible spontaneous sputum specimens can be difficult to use due to the high squamous cell contamination and a high proportion of necrotic cells (180). Induced sputum has risen in popularity as it is felt to reflect inflammation of the main airways and therefore provides similar but not identical information to bronchial washes and bronchoalveolar lavage (181-183). However induced sputum does not correlate well with bronchial biopsy findings (181, 182). With increasing recognition of the potential of induced sputum as a non-invasive measure, methods for the technique have been refined with the aim of employment in clinical trials (180, 181, 184, 185). There is currently a debate as to the role for induced sputum monitoring in the management of asthma but it is unlikely to be employed outside specialist secondary care asthma clinics (48).

1.9.3 Induced sputum methodology

1.9.3.1 Induction method

The common thread in all sputum induction protocols is the inhalation of nebulised sterile saline from a high output ultrasonic nebuliser, the need for the administration of pre induction bronchodilator and for regular monitoring of the participant's symptoms and FEV_1 during the induction period (181, 184). Beyond this there is a difference in opinion as to the optimal method. A range of time periods and concentrations of sterile saline have been employed by different centres and by multicentre studies. Some centres expose subjects to nebulised saline for periods of five minutes and others use periods of seven or more minutes. There is also some variation with regards the concentration of saline used with some groups increasing the saline concentration with each new inhalation period and others keeping a constant concentration. Our group currently employs seven minute inhalation periods and 3, 4 & 5% saline. The subject moves onto the next highest concentration if their lung function allows (180, 181).

1.9.3.2 Sputum processing

The processing of induced sputum samples has also been the cause of some debate with some groups advocating the use of the whole sample and others selection of sputum plugs (186). A recent working group concluded that both methods of selection are useful for differentiating health and disease but were felt to not be interchangeable and it was advised to keep to one method for the duration of a trial (186). Our group currently employs the whole sample method. Once processed a sample of the sputum is added to a slide and processed for counting. The current consensus is that a minimum number of 400 non-squamous cells are required for a representative count (186). The count should consist of a total cell count, squamous cell count and a differential of non-squamous cells with samples being discarded when the percentage of squamous cells is greater than 80% (187). Induced sputum results expressed as a percentage of non squamous cells bypasses concerns about dilution in the whole sample method and allows for good reproducibility (187, 188).

1.9.4 Induced sputum-Clinical trials

Induced sputum has been demonstrated to be highly reproducible in asthma (180), to respond appropriately to allergen challenge and corticosteroid treatment (181) and to correlate with bronchial provocation testing and exhaled nitric oxide levels in adults (189) and children (190) (although these findings are not consistent (191, 192)).

1.9.4.1 Induced sputum-Eosinophilia

Induced sputum facilitates sub-categorisation of asthma into groups that respond to conventional treatments based on the predominance of certain cell types (essentially as an extension of previous work in spontaneous sputum (179)). Therefore induced sputum has become employed as a study endpoint in cross sectional and intervention trials. Treating subjects with sputum eosinophilia with corticosteroids to reduce the percentage of eosinophils below a pre-set target has been demonstrated to result in a greater improvement in asthma control relative to standard clinical measures (193) whilst the absence of sputum eosinophilia indicates that corticosteroid dose reduction can be performed safely

(193). The degree of sputum eosinophilia also negatively correlates with FEV_1 (194).

1.9.4.2 Induced sputum-Neutrophilia and paucicellular sputum

Sputum neutrophilia is associated with a reduced response to corticosteroid treatment in asthma as a raised sputum neutrophil count has been described in smokers with asthma (41, 42) and subjects with severe asthma (195-199). In prospective clinical trials sputum neutrophilia has also been demonstrated to correlate with steroid resistant inflammation (44, 46).

In subjects with raised sputum neutrophils there is evidence of an inverse correlation with FEV_1 (194, 200) and irreversible airflow obstruction (200). In some subjects with asthma an induced sputum profile is observed which has neither a raised eosinophil nor neutrophil count. This group has been described as 'paucicellular' or 'paucigranulocytic' and appears to indicate a milder form of asthma as it is associated with better asthma control (46).

1.9.4.3 Induced sputum-Definition of eosinophilia and neutrophilia

The definition of sputum eosinophilia has been developed through research examining sputum profiles from normal subjects. This work has resulted in the current consensus for the cut off being an eosinophil percentage of >2% (44, 46, 201, 202). The definition of neutrophilia is slightly more problematic as the sputum neutrophil percentage increase with age (200, 202). A correction based on subject age may enable further examination of the importance of sputum neutrophilia (194). A pragmatic approach that has been advocated is to define neutrophilia as a sample with greater than 50% neutrophils. However the need for research addressing normal ranges for induced sputum is clear and a call for further work has recently been made (203).

1.9.4.4 Induced sputum-Reproducibility

Induced sputum demonstrates good reproducibility with intraclass correlation co-efficients (ICC) for eosinophils of 0.85 and neutrophils of 0.57 (187) for whole sputum sampling. Selected sputum processing has been associated with ICCs of

0.63 for eosinophils and 0.57 for neutrophils (204) in one study and 0.94 for eosinophils and 0.81 for neutrophils in another (180).

1.9.5 Sputum supernatant cytokines

Investigation of the possible mechanisms responsible for the development of asthma and allergy in animal models, *in-vitro* systems and clinical studies has led to the identification of pathways in the innate and adaptive immune system that may be activated in asthma. The cytokines and chemokines associated with these pathways can be detected in the supernatant phase of sputum samples obtained from patients using immunological techniques.

In recent years the number of antibodies available for the cytokine/chemokine of interest has increased and it is now possible to detect multiple signals within the same sample using commercially available systems. Therefore researchers are able to examine the symphonic orchestration of the immune response in contrast to the limited and potentially biased examinations in the past. Using these new approaches it should be possible to determine the relative contribution from the innate and adaptive immune arms of the immune system.

1.9.5.1 Effect of sputum processing on supernatant cytokines

However the examination of cytokines and chemokines in induced sputum presents some unique difficulties due to the use of the reducing agent dithiothreitol (DTT) (181, 205-207). DTT is routinely employed in the preparation of induced sputum as it reduces the viscosity of the sample enabling easy removal of sputum plugs. An unfortunate side effect is loss of antigenicity of some cytokines due to the disruption of thiol bonds within their structure (205, 206). Recent work employing removal of DTT post sputum processing using dialysis cassettes (206), removal of sputum supernatant prior to addition of DTT or use of lower concentrations have been attempted with improvements in detection levels. Despite the availability of techniques to examine cytokines in induced sputum the identification of a consistent sputum cytokine profile predictive of a treatment response remains elusive perhaps as a result of this issue.

1.9.6 Exhaled markers of inflammation-Nitric oxide

Nitric oxide (NO) is abundant in the human body, where it has many roles including vasomotor control and neurotransmission. NO is produced from L-arginine by nitric oxide synthases (NOS) through oxidative conversion (208). There are three forms of NOS and two of the three are constitutively expressed-neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). Both nNOS and eNOS are activated by increased intracellular calcium as a result of their calmodulin binding region. nNOS has a role in non-cholinergic non-adrenergic bronchial smooth muscle relaxation and eNOS in bronchial epithelial ciliary beat frequency (208). The third NOS, inducible nitric oxide synthase (iNOS), is upregulated by immunological and inflammatory stimulation, is relatively insensitive to intracellular calcium levels and produces much larger amounts of NO compared to nNOS and eNOS (208).

NO is present in exhaled breath and was first detected in humans in 1991 (209) and in subjects with asthma soon after (210). Expelled air from the lungs does contain NO but it is present at much higher levels in the nose and paranasal sinuses (210). However with adherence to good measurement techniques nasal NO can be excluded through closure of the soft palate in the majority of subjects (47). Bronchial epithelial cells, airway smooth muscle, macrophages, neutrophils and alveolar cells all express iNOS and contribute to the production of exhaled NO but the bronchial epithelium is responsible for the lion's share (208, 211, 212). The role of nitric oxide in the lung is complex as it is highly reactive and it can form reactive nitrogen species such as peroxynitrite, react with cysteine residues on proteins to form S-nitrosothiols and hence interfere with zinc finger motifs of transcription factors, interact with guanylate cyclase synthase to increase intracellular cGMP levels and alter mitochondrial metabolism (208, 211, 212).

1.9.7 Nitric oxide in asthma

The ability of iNOS to be induced by inflammation coupled to the effect of increased nitric oxide on cellular processes and the observations that exhaled nitric oxide is increased in asthma exacerbations and reduces with steroid

treatment has led to an interest in the monitoring of exhaled NO in asthma. Exhaled NO has subsequently become established as an endpoint for the assessment of new therapies targeted against inflammation. In recognition of this recent consensus guidelines have provided expert guidance aiding both comparison of studies and avoidance of errors in assessment (47). Unfortunately the specificity of raised single flow measurement of NO for asthma is less than desired as production is increased in other inflammatory conditions, for example cirrhosis (213), systemic lupus erythematosis (214), COPD (215) and lung transplant rejection (216).

1.9.7.1 Employment in asthma control algorithms

However there are also problems with exhaled NO and its utilisation in the management of asthma which has led to questions over its usefulness in clinical research. Several groups have explored the utility of exhaled NO in the management of asthma (217-219) but no reduction in asthma exacerbations occurred when exhaled NO was used to guide management. In the largest management trial to date (219) the use of NO in conjunction with clinical assessment resulted in increased inhaled corticosteroid usage without improvement in asthma control, lung function, unscheduled visits or need for hospitalisation.

1.9.7.2 Reference ranges

Measurement of NO using one standard exhaled flow rate also suffers from less than ideal sensitivity and specificity. There is difficulty with cut offs given the degree of overlap that exists between subjects with asthma and normal subjects. However a NO level of <16ppm (measured at 50ml/sec) appears to indicate of an absence of eosinophilia and >26ppm correlates with sputum eosinophilia (albeit weakly) (213, 215). However other cut offs have been employed (219) and one manufacturer suggests that a value of greater than 50 ppb in adults is suggestive of eosinophilic inflammation (Aerocrine AB, Sweden). I would agree that similar to induced sputum there is an urgent need for research addressing normal values for this non-invasive marker (203).

1.9.7.3 Effects of cigarette smoking

Another major problem for the employment of exhaled NO in research and clinical practice is the effect of current smoking on exhaled NO levels. Smoking acutely increases exhaled nitric oxide levels, reflecting the concentration of nitric oxide within the cigarette (51, 54) and chronic smoking results in a reduction of exhaled NO with measurements in smokers with asthma usually being in the normal range (50, 220). However a recent study does suggest that a percentage change in the exhaled NO concentration may reflect changes in asthma control and therefore may overcome some of the problems with the use of this endpoint in smokers with asthma (221).

The mechanism responsible for the reduced exhaled NO concentration displayed by this group has been debated. Some authors propose that the high concentration of NO in cigarette smoke could be inhibiting iNOS (52, 54) and others that the increased oxidative stress results in consumption of airway NO (51, 52). A recent study comparing smokers with asthma to non smokers with asthma demonstrated equal levels of iNOS in the smokers and non smokers with asthma but raised arginase 1 and ornithine de-carboxylase expression in smokers suggesting that substrate competition may be contributing to the reduced NO levels in smokers with asthma (53).

1.9.7.4 Extended flow nitric oxide analysis

Early work examining exhaled nitric oxide noted that the nitric oxide concentration level in exhaled breath varied with the flow rate of exhalation with an inverse exponential relationship between flow rate and exhaled nitric oxide concentration being evident (222). The production rate of NO (V_{NO}) also increases linearly with the rate of exhalation similar to heat exchange in a pipe. These characteristics of exhaled NO led researchers to develop models based on the principle that the lungs could be divided into two compartments or phases; a fixed volume conducting airways region and an expandable alveolar region.

This two compartment model allows for the derivation of estimates for alveolar NO levels (C_{alv}, ppb), airway wall NO diffusion (D_{aw}, pl/s/ppb), airway wall nitric oxide flux (J_{aw}, pl/s) and airway wall NO concentration (C_{aw}, ppb) depending on

the flow rates used and the regression model employed. Plotting nitric oxide production against flow rate for a variety of flow rates allows derivation of these parameters using linear and non linear regression. This alternative approach to the assessment of exhaled NO has led to hopes that extended flow analysis will increase the sensitivity and specificity of exhaled NO. Alveolar NO levels may reflect inflammation in the smaller airways (223) and recent work has employed this endpoint in the examination of new formulations of inhaled corticosteroids in the hope of detecting a reduction in small airway inflammation (224). Alveolar NO also appears to be unaffected by smoking so could be useful as a measure of inflammation in COPD and smokers with asthma (225, 226). The technique may also be able to improve the specificity of exhaled NO through the definition of extended flow profile 'signatures' for different conditions. For example, different conditions may have different rates of airway wall NO production, airway wall diffusion and alveolar and airway NO levels. Steroid naïve non-smoking asthmatics have raised alveolar NO, diffusion and flux compared to steroid treated asthmatics who display raised diffusion only (227). Extended flow rate nitric oxide measurement has not been studied in smokers with asthma and given the absence of an impact of smoking on alveolar NO in normal smokers and smokers with COPD extended profile analysis may provide a useful non-invasive marker of inflammation in smokers with asthma.

1.9.8 Exhaled breath condensate

Exhaled breath contains vapours and aerosols that can be analysed by collecting via cooling and precipitation (228, 229). This phase of exhaled breath, known as exhaled breath condensate (EBC), has been utilised since the early nineteen eighties as a non-invasive method of examining airway lining fluid and its chemical environment composition. EBC has been demonstrated to contain non-volatile compounds such as cytokines, lipids, surfactant, ions, oxidation products, adenosine, histamine, acetylcholine and serotonin and over 200 volatile compounds such as ammonia, H_2O_2 and ethanol can be detected (229, 230). EBC has subsequently been used to examine a number of disease states (for example asthma, cystic fibrosis and bronchiectasis) and their response to treatment (228, 231-233).

1.9.8.1 Exhaled breath condensate-methodological considerations

EBC gained interest as it was initially thought to have the potential to obtain equivalent samples to invasive procedures such as bronchoscopy and broncho-alveolar lavage and non-invasive procedures that require specialist processing such as induced sputum. However there are methodological issues that need to be addressed and hence concerns over the applicability of EBC data. For example, EBC has recently been demonstrated to have poor correlation with bronchoalveolar lavage for a number of measurements (234). There is also a need for a reliable marker to calculate the degree of dilution of exhaled biomarkers by water in the EBC (235-237). Work is currently ongoing to develop a suitable reference marker.

1.9.8.2 Exhaled breath condensate pH

Another avenue of examination in exhaled breath condensate is EBC pH. Hunt et al have demonstrated that asthmatics admitted with acute exacerbations have a reduced EBC pH that normalises within forty eight hours of treatment (228). EBC pH has subsequently been examined in a number of conditions including COPD (232), bronchiectasis (238) and cystic fibrosis (239, 240). Measurement of pH after de-aeration with argon has been demonstrated to be consistent day to day, week to week and person to person and to be stable over a range of temperatures of collection and for 2yrs in storage (232, 241). Similarly EBC pH is unaffected by hyper and hypoventilation (242) and methacholine induced bronchoconstriction (241).

1.9.8.3 Mechanisms responsible for EBC acidification

What causes the airway acidification demonstrated by EBC pH? One potential mechanism is alteration of airway cell numbers and proportions. Both neutrophils and eosinophils can cause airway acidification. Neutrophils can form hypochlorous acid via the myeloperoxidase catalysed reaction between H_2O_2 and chloride. Eosinophils can produce acid via eosinophil peroxidase which catalyses a reaction between H_2O_2 and halides to form hypohalous acids. Hunt et al did not examine induced sputum in their study so we do not know the relative contribution of either cell to the drop in pH observed in that trial. However

induced sputum neutrophilia has demonstrated a strong negative correlation with EBC pH in subjects with COPD and bronchiectasis and a strong negative correlation between sputum eosinophilia and pH in subjects with asthma (238). Further research comparing smokers and non-smokers with asthma has demonstrated that smoking is associated with a lower EBC pH (58). No difference was present in percentage neutrophil counts between the two groups in this study.

An alternative mechanism for airway acidification in asthma could be alterations in the airway epithelium. Activation of cell surface exchange pumps such as Na^+-H^+ exchange protein 1 and anion exchange proteins 1 and 3 in the context of reduced airway lining fluid buffering have been suggested as potential causes of acidification of inflamed airways (243). Other potential mechanisms for EBC acidification include inappropriate collection of gastric air & micro aspiration of gastric contents.

1.10 Hypotheses and aims

The overall aim of this thesis was to examine two potential new therapies for smokers with asthma and to gain understanding of the altered inflammatory processes present in this group.

The following hypotheses were examined in the two studies conducted for this thesis:

1.10.1   Theophylline & Rosiglitazone

- Low dose theophylline will restore corticosteroid sensitivity in smokers with asthma due to a restoration of HDAC activity

- The oral PPARγ agonist rosiglitazone will demonstrate superiority to low dose inhaled beclometasone

1.10.2   Corticosteroid sensitivity study

- Smokers with asthma display a muted lung function response to an oral corticosteroid trial compared to non-smokers with asthma

- Smokers with asthma have an altered pulmonary and systemic cytokine environment compared to non-smokers with asthma

- Smokers with asthma display a reduced sputum and blood total HDAC activity compared to non-smokers with asthma

- Smokers with asthma display alterations in flow independent nitric oxide parameters compared to non-smokers with asthma

2 Methods

2.1 Regulatory approval

The studies presented in this thesis were reviewed and approved by the West Glasgow Ethics Committee. The study examining the efficacy of rosiglitazone in smokers with asthma was also submitted for review and approval by the Medicines and Health Regulatory Authority. All subjects received an information sheet and attended for a discussion of the associated study protocol prior to consent and enrolment.

2.2 Recruitment methods

The majority of patients that took part in the studies presented in this thesis were recruited from general practices in the Greater Glasgow and Clyde and North and South Lanarkshire Health Board areas. The practices were approached by letter and those willing to participate were visited by a member of the study team who undertook a search of the practice records to identify potential recruits, taking care to exclude subjects who failed to meet entry criteria. A list of potential recruits was then left with the practice for review and vetting to prevent inappropriate approaches. Once this process was completed a further visit to the practice was arranged where the study team member produced an approach pack containing an invitation letter, a response form and a stamped address envelope. The practice then posted the approach pack to the potential recruits in their normal mail. Interested subjects who contacted the asthma research unit were vetted by phone call and those who were deemed suitable were invited to discuss the study.

Commercial adverts approved by the local ethics committee were also used to help recruitment to the theophylline and rosiglitazone study. Two radio advertisement campaigns were utilised along with a month of posters placed at major transport hubs in the Glasgow area. A small number of subjects who had previously participated in trials run by the Asthma Research Unit were also suitable and agreed to participate after approach by letter.

2.3 Study design

2.3.1 Efficacy of theophylline and rosiglitazone in smokers with asthma

This study was a randomised, prospective, double-blind, double-dummy, active comparator, parallel group design. Mild to moderate (2) stable asthmatics aged 18 to 60 on ≤1000 mcg beclometasone (or equivalent) per day who were regular smokers of ≥5 cigarettes per day and with a pack year history of ≥5 pack years were eligible for enrolment. Subjects who were willing to participate were offered smoking cessation advice and those unwilling to quit smoking at that time and did not have conditions excluding participation were enrolled.

All subjects had to demonstrate reversible airflow obstruction with a FEV_1 bronchodilator response of ≥12% (and >200ml). Subjects were also monitored for asthma stability for up to six weeks and underwent a corticosteroid weaning and monitoring phase that lasted one month within this period. Subjects were excluded from randomisation if they experienced an exacerbation of asthma at any point during this run-in phase. An asthma exacerbation was defined as a reduction in morning peak flow (PEF) of 30% or more from baseline or asthma exacerbation requiring oral corticosteroids or hospitalisation or emergency department/general practice visit or the presence of asthma symptoms deemed unacceptable to either the study co-ordinator or study subject.

Other exclusion criteria included:

- Asthma exacerbation or respiratory tract infection within six weeks of screening

- Additional respiratory condition e.g. bronchiectasis

- Plan to reduce or stop cigarette smoking

- Pregnancy or plan to conceive

- Diabetes mellitus

- Recent myocardial infarction/unstable angina or any history of cardiac failure (past 6 months)

- Anaemia or abnormal renal or hepatic laboratory values

- Contraindication to treatment with either theophylline, rosiglitazone or inhaled corticosteroid

- Recent drug or alcohol abuse

- Morbid obesity (defined as BMI>40)

- Inability to perform spirometry

- Requirement for treatment with any other asthma medications (except inhaled salbutamol and allocated trial medication) from screening until study completion.

At the end of a two-week inhaled corticosteroid free period each subject attended for a randomisation visit, which comprised spirometry and PEF recordings, completion of an asthma control questionnaire (ACQ)(244), induced sputum for differential count, supernatant and sputum macrophage HDAC activity assay and routine bloods for safety (full blood count, renal and liver function testing) and characterisation (total and specific IgE, total cholesterol, LDL, HDL and triglycerides levels).

Subjects were then randomised to either 100mcg twice a day inhaled hydrofluoroalkane beclometasone dipropionate (Qvar©, IVAX, Runcorn, Cheshire, UK) [Equivalent to approximately 400mcg per day chlorofluorocarbon beclometasone] (245), 4mg twice daily oral rosiglitazone (Avandia®, GSK, Greenford, Middlesex, UK), 200mg twice daily oral theophylline (Uniphyllin® Continus®, NAPP, Cambridge, UK) or 200mg twice daily oral theophylline in combination with 100mcg twice a day inhaled hydrofluoroalkane beclometasone dipropionate. Subjects returned for pre-bronchodilator lung function at two weeks and repeated the assessments carried out at the baseline visit (spirometry pre and post bronchodilator, peak flow, induced sputum and supernatant, blood and serum & ACQ) after four weeks. This visit was performed at the same time of day (+/- 2 hours) as the baseline visit. Continued regular smoking was confirmed through the measurement of exhaled carbon monoxide and urinary cotinine metabolites. Subjects were instructed to omit smoking for three hours

prior to each study visit and were informed that if they did not adhere to this request their visit would be re-scheduled and where this was not possible they would be excluded from the study for non-compliance. If an excessively raised carbon monoxide level was detected subjects were challenged over their compliance with abstinence from smoking.

The primary endpoint was change in pre-bronchodilator FEV_1 from baseline to day 28. Secondary endpoints were change from baseline to day 28 in pre-bronchodilator PEF, FVC, FEF_{25-75}, FEF_{75} & asthma control questionnaire score. Exploratory endpoints included sputum differential count, sputum cytokine profile & sputum macrophage histone deacetylase activity (HDAC).

2.3.2 Determinants of corticosteroid insensitivity in smokers with asthma

The study was a cross-sectional design with open label, unblinded use of oral dexamethasone. Smokers, ex-smokers and non-smokers with mild to moderate asthma were recruited to the study. Subjects were allowed treatment with up to a maximum of 2000 mcg beclometasone (or equivalent), long acting β_2 agonists and leukotriene receptor antagonists. Theophylline was withdrawn (if prescribed) for 6 weeks prior to the start of the steroid trial and restored at the end of the study. Smokers with asthma were eligible if they were currently smoking ≥5 cigarettes per day and had a ≥5 pack year history. Ex-smokers with asthma were eligible if they had ceased smoking 2 or more years from the date of recruitment and had a ≥5 pack year history. Non smoking subjects were required to have no history of regular smoking and to be current non-smokers. All subjects performed urine cotinine and exhaled carbon monoxide testing at each visit to confirm smoking status. Smokers with asthma were instructed to omit smoking for 3 hours prior to each visit and were informed that if they did not adhere to this request their visit would be re-scheduled and when not possible they would be excluded from the study for non-compliance. If an excessively raised carbon monoxide level was detected subjects were challenged over their compliance with abstinence from smoking.

All recruited subjects had to demonstrate either reversible airflow obstruction with a FEV_1 bronchodilator response of $\geq 12\%$ (and >200ml), PEF lability or a positive methacholine test to be eligible to perform the oral corticosteroid trial. If subjects did not demonstrate a positive FEV_1 bronchodilator response then they returned after completing a PEF diary. If this did not demonstrate PEF lability then a methacholine test was performed. Baseline measurements included spirometry and PEF, exhaled nitric oxide measurement, exhaled breath condensate and induced sputum collection, asthma control questionnaire score and blood for HDAC activity and cytokine assessment. Corticosteroid sensitivity was assessed using a two week trial of oral dexamethasone, adjusted for body surface area. Each subject was allocated a daily dose of 6mg/1.74 m^2 oral dexamethasone and steroid response was assessed as the change in pre-bronchodilator FEV_1 at two weeks. The assessments carried out at baseline were also repeated with the inclusion of blood cortisol to check compliance with corticosteroids. If blood cortisol was suppressed below 50nmol/l then subjects were deemed to be compliant with therapy and their post steroid trial data was assessed. This visit was performed at the same time of day (+/- 2 hours) as the baseline visit. A subset of subjects returned at one month to repeat some of the assessments to determine the duration of oral corticosteroid effect.

Exclusion criteria were:

- Patients with unstable asthma; defined as the presence of 1 or more of the following events in the month prior to study [Emergency/'out of hours' visit to GP for asthma exacerbation; GP visit to patient at home for asthma exacerbation or A & E attendance/hospital admission for asthma exacerbation]

- Treatment with oral corticosteroids in the past month

- Need for maintenance oral corticosteroid therapy

- Need for treatment with theophylline for course of study

- Pregnancy or planning to become pregnant over course of study

- Presence of medical condition likely to be exacerbated by treatment with oral corticosteroids

- Plan for smoking cessation or reduction during course of study

2.4 Demonstration of eligibility for recruitment to trials

2.4.1 Reversibility testing

Improvement in airflow obstruction in response to inhaled bronchodilator in a subject with a history consistent with asthma is accepted as diagnostic for the condition (1, 2) and is a frequently used entry criteria for clinical trials.

In both trials reversibility testing was performed in all subjects by administering 2.5mg of nebulised salbutamol for five minutes following suitable baseline spirometric recordings. Subjects then performed spirometry from 30 minutes post nebuliser. A minimum of three acceptable efforts were obtained and a maximum of eight efforts was allowed to meet this criterion. The highest FEV_1 and FVC obtained were used for analysis. Subjects were asked to withhold inhaled and oral treatments according to consensus recommendations (table 2.1) (246). Reversible airflow obstruction was defined as an improvement in FEV_1 of 12% or greater (and 200ml or more in volume) and was calculated using the formula:

$$\% \text{ reversibility} = ((\text{post bd } FEV_1 - \text{pre bd } FEV_1)/ \text{ pre bd } FEV_1) * 100$$

where bd = bronchodilator

Treatment	Withdrawal period
Short acting inhaled bronchodilator	4 hours
Long acting inhaled bronchodilator	24 hours
Oral bronchodilator	24 hours

Table 2.1 Withdrawal periods for bronchodilators prior to reversibility testing. Based on (246).

2.4.2 Peak flow lability

Spontaneous variation in peak flow is recognised as a defining characteristic of asthma and is a reflection of the variable nature of the dyspnoea experienced by some subjects with asthma (1). Entry to the trial examining the corticosteroid responses of smokers, ex-smokers and non-smokers with asthma was therefore permitted if variable airflow obstruction was demonstrated through peak flow recordings if spirometric reversibility testing was negative at the first visit.

Subjects were issued with a diary card and peak flow meter (EN 13826, Clement Clarke, Harlow, UK) and were instructed to perform three adequate efforts in the morning and evening and to record the highest recording in their diary for one to two weeks prior to review. The threshold of peak flow lability required for entry to the trial followed published methodology i.e. 20% variation in amplitude over 3 days in the period of diary recording (with a minimum change of 60 litres)(1).

2.4.3 Bronchial provocation testing

The demonstration of bronchial hyperreactivity in a subject with a high pre-test probability of asthma is accepted as diagnostic for the condition (247) and this approach is frequently applied as an eligibility criteria in clinical research where subjects cannot demonstrate peak flow lability or significant FEV_1 improvement post nebulisation due to good asthma control. Testing for the presence of bronchial hyperreactivity centres on the administration of a challenging agent, most commonly histamine or methacholine, in serial doubling doses via a modified Wright's nebuliser (Airlife Sidestream® high efficiency nebuliser, Cardinal Health, UK) calibrated to supply 0.13ml/min of solution.

In the study examining the corticosteroid responses of smokers, ex-smokers and non-smokers with asthma participation was allowed if subjects had a positive methacholine test and a history compatible with asthma. Non-smokers and ex-smokers with asthma were allowed to perform methacholine testing if their FEV_1 was greater than or equal to 60% predicted. However smokers with asthma were required to have a pre bronchodilator FEV_1 of greater than or equal to 80%

predicted to minimise recruitment of subjects with chronic obstructive pulmonary disease.

In preparation for the test subjects were asked to omit asthma medications, anti-histamine medications and foods and drinks containing caffeine as per recommendations (247)(table 2.2). Female subjects underwent pregnancy testing and were only allowed to perform methacholine testing if not pregnant.

Treatment	Duration of withdrawal (hours)
Short acting β_2 agonist	8
Long acting β_2 agonist	48
Short acting anti-muscarinic	24
Long acting anti-muscarinic	48
Leukotriene receptor antagonists	24
Theophylline	48
Anti-histamines	48
Caffeine containing food or drinks (chocolate, tea, coffee, soft drinks)	Day of study

Table 2.2 Advised durations of withdrawal from medications and foods.
Table adapted from (247).

Subjects initially performed pre-challenge spirometry to determine their best pre-challenge FEV_1. Nebulised saline was then administered as an initial challenge for two minutes. The target drop of 20% was calculated from the highest post saline FEV_1 following repeat spirometry. The subject then inhaled increasing doses of methacholine in two minute dosing periods by tidal breathing whilst wearing a nose clip. On completion of each nebulisation phase spirometry was performed at 30, 90 and 180 seconds. If the subject's FEV_1 did not drop by 20% or more in response to the methacholine dose then this was followed by a subsequent dose of methacholine until the subjects FEV_1 declined by 20% from their highest post saline measurement or the final dose (16mg/ml) was completed. The dose of methacholine used started at 0.03mg/ml and this was

followed by 0.0625mg/ml, 0.125mg/ml and then on in doubling doses up to 16mg/ml. Three millilitres of each methacholine solution was placed in the nebuliser just prior to administration. Methacholine was obtained from the hospital pharmacy (Western Infirmary & Gartnavel General Hospital, Sterile Production Unit) and was kept refrigerated to maintain stability. New stock was supplied every six months.

The provoking concentration of methacholine required to produce a fall in FEV_1 by 20% (PC_{20}) for each subject was calculated by linear interpolation using the formula:

$$PC_{20} = \text{antilog}\left[\log C_1 + \frac{(\log C_2 - \log C_1)(20 - R_1)}{R_2 - R_1}\right]$$

C_1 = second to last methacholine concentration, C_2 = last methacholine concentration, R_1 = % fall in FEV_1 after C_1, R_2 = % fall in FEV_1 after C_2

Figure 2.1 Methacholine calculation method. Table adapted from (247)

A PC_{20} of <8mg/ml in the context of a clinical history consistent with asthma, appropriate symptoms during the test (chest tightness, dyspnoea) and good quality spirometric efforts was considered to confirm the diagnosis of asthma and eligibility for entry to the trial.

2.4.4 Urine cotinine

Smoking history was confirmed by measurement of nicotine metabolites in a specimen of the subjects urine using the SmokeScreen[TM] sampling system (GFC Diagnostics, Stourbridge, UK). The SmokeScreen[TM] system detects the metabolised derivatives of nicotine through their reaction with diethylthiobarbituric acid in the sampling system as this turns the urine pink. This method removes the problems associated with the measurement of urine cotinine alone such as conversion to other metabolites.

The level of nicotine metabolites present was objectively assessed using the SmokeScreen[TM] colorimeter (GFC Diagnostics, Stourbridge, UK). The colorimeter takes a baseline reading after mixing the sample and then repeats the

measurement 5 minutes later. Previous research has demonstrated that the reaction of nicotine metabolites occurs in a predictable linear fashion and hence the change in colour over time can be converted to concentration of nicotine metabolites (248). The colorimeter performs this calculation and displays a 'cotinine-equivalent' concentration (0-20 µg/ml) and a category of 'smoking' (non-smokers, passive smoker, mild, moderate and severe smoker). The cotinine equivalent concentration ranges for each category are non-smokers (0.0-0.3 µg/ml), passive smoker (0.4-1.0), light smoker (1.1-5.0), medium smoker (5.1-10.0), heavy smoker (10.1-15.0) and v heavy smoker (15.1-21.0).

2.4.5 Exhaled Carbon Monoxide measurement

All subjects performed exhaled carbon monoxide at each timepoint in both studies to provide substantiation to their assertion that their smoking history was unchanged. Exhaled carbon monoxide was detected using a Pico Smokerlyser® (Bedfont Scientific Ltd, Rochester, UK). The Smokerlyser® detects exhaled carbon monoxide using an incorporated electrochemical sensor and presents the result as percentage carboxyhaemoglobin, parts per million and as a 'traffic-light' read out. The concentration range was 0-80 parts per million with an accuracy of +/- 2%. The non-smoking range was defined as 0-7 parts per million and current smoking 8 parts per million and above (249).

Previous research has demonstrated that it takes 2-8 hours for the carbon monoxide level to reduce by half (250-252) and at least 24 hours of smoking cessation is required for a smoker's carbon monoxide level to return to that of a non-smoker (252). A grossly elevated level of carbon monoxide resulted in questioning of the volunteer with regards their compliance with omission of smoking for the visit. Non-smokers and ex-smokers had to demonstrate levels consistent with no current smoking. Smokers had to demonstrate elevation of their carbon monoxide level consistent with current smoking.

Subjects performed three readings and the mean was used for analysis. Subjects inhaled and held their breath for 15 seconds and, when prompted by the Smokerlyser®, exhaled completely into the D-piece valve via a cardboard mouthpiece. Both the valve and mouthpiece are single use only. The valve contains a filter that prevents false readings due to alcohol and other organic

compounds. Calibration was carried out every six months using a cylinder of carbon monoxide certified at 20 parts per million (Bedfont Scientific Ltd, Rochester, UK).

2.5 Clinical endpoints

2.5.1 Spirometry

Spirometric recording was performed in all subjects using electronic pneumotachograph spirometers (Vitalograph Ltd, Maids Moreton, Buckingham, UK) to the standards set by the joint ATS/ERS guidelines on spirometry (253). Calibration of each spirometer was carried out every day prior to first use with a 3 litre reference syringe with adjustment for ambient temperature (253). Servicing was also carried out on an annual basis by Vitalograph technicians.

Spirometric manoeuvres were consistent with published recommendations (253). To ensure consistent and valid performance of spirometry subjects observed a demonstration by the study doctor or research nurse prior to their first attempt. The subject was then instructed to take a maximum breath in and to then immediately place the mouthpiece in their mouth and blow out with maximum effort into the mouthpiece until no further air could be expelled. Active encouragement to continue exhalation until a suitable effort had been performed was provided by the supervising staff member. The procedure was repeated until three acceptable manoeuvres were available ensuring proper understanding of the technique and consistency in performance. A maximum of eight efforts was allowed to facilitate this process. An acceptable exhalation manoeuvre was defined as one which demonstrated a good rapid start, was free from artefact (cough, sub-maximal effort, glottis closure etc) and that had a satisfactory duration of exhalation. The duration was satisfactory if a plateau was reached and a minimum of six seconds of exhalation had been performed. Inter-manoeuvre variability was reduced by accepting efforts where the last two FEV_1 results did not vary by more than 5% or 150ml. The highest FEV_1, FVC, FEF_{25-75} and FEF_{75} efforts were recorded for analysis.

2.5.2 Peak expiratory flow

All patients were required to perform peak flow measurement at visits where spirometry was performed. Clement Clarke meters with EU scale EN 13826 were used for all measurements (Clement Clarke, Harlow, UK). PEF procedure was first demonstrated by the study nurse or doctor and subjects were then asked to stand upright and blow with maximum effort into the peak flow meter. The highest of three acceptable readings was recorded.

2.5.3 Asthma Control Questionnaire

The Juniper Asthma Control Questionnaire (ACQ) is a simple, reliable and sensitive questionnaire that was developed to allow quick assessment of asthma control in all severities of asthma and has been demonstrated to effectively demonstrate the impact of asthma treatment interventions (244).

The score produced by the questionnaire is based on a series of seven questions. The first six questions cover the symptoms that the respondent has experienced in the past week with regards night time wakening, limitation of normal daily activities, early morning wakening, dyspnoea and wheeze and frequency of use of inhaled β_2 agonist. Each question is answered by the respondent selecting one choice from six and the severity of choices ranging from responses which signal no symptoms or none to maximum severity for that particular symptom. The final question is answered by the clinic staff using the respondent's FEV_1 result from spirometry performed on the day of the assessment.

The respondent's score is the mean for all seven fields, resulting in maximum control being represented by a score of zero and the worst level of control a score of six. Recent research has determined that a score less than 0.75 is indicative of good asthma control, a score of greater than 1.5 indicative of inadequate control and a change in subjects ACQ score of 0.5 or more is considered clinically significant (254).

2.5.4 Exhaled Nitric Oxide

Exhaled nitric oxide ($F_{E_{NO}}$) was measured at multiple flow rates (30, 50, 100, 150, 200, 250 & 300ml sec^{-1}) using a Niox-Flex analyser (Aerocrine AB, Sundbybergsvägen 9, SE-171 73 Solna, Sweden) which meets joint ATS/ERS criteria for the measurement of on-line $F_{E_{NO}}$ (47).

The Niox-Flex measures $F_{E_{NO}}$ by chemiluminescence. Briefly the principle of detection involves the subject's $F_{E_{NO}}$ reacting with ozone produced within the machine. This generates nitrogen dioxide with electrons in an excited state i.e. that are occupying a higher energy level than normal. Subsequent return of the electrons to their normal excitation level is associated with the discharge of electromagnetic radiation at a wavelength of 600-3000nm. This is detected by a photomultiplier tube incorporated within the machine and as a linear relationship exists between the level of radiation emitted and the $F_{E_{NO}}$ exhaled enables derivation of $F_{E_{NO}}$. The Niox-Flex has a published measuring range of 0-200 ppb, a detection limit of 1 ppb, a sampling frequency of 20Hz, a response time of <1.5 seconds and an accuracy of +/- 2.5 ppb for levels < 50 ppb and +/- 5 % of values >50 ppb. Calibration was carried out every two weeks or as required.

Prior to performing the test subjects received an explanation on how to correctly perform the test from the supervising doctor or nurse. Following this the subject performed the test according to consensus guidelines (47). The Niox-Flex requires subjects to take a deep inspiration both in and then exhale out through the machine's mouthpiece. This ensured scrubbing of inhaled air which passed through the mouthpiece to the subject. The Niox-Flex automatically calculates nitric oxide output (V_{NO}) and exhaled nitric oxide concentration, discarding measurements inconsistent with previous results at that flow rate and which did not demonstrate a plateau. Visual feedback was provided by the machine to maintain exhalation pressure above that required for closure of the velum, reducing nasal nitric oxide contamination.

The results obtained from the multiple flow rates performed by the subjects in the Niox-Flex were used to calculate estimates for extended flow parameters for each subject based on modelling equations from previously published research (52, 222, 227). Extended flow analysis allows the derivation of several estimates

of nitric oxide metabolism including alveolar nitric oxide as a result of modelling of the lung as a compartment divided into two parts. The first consists of a non-expandable conducting airway which corresponds to the trachea to division 16 of Weibel's model of the airways and an expandable alveolar region that comprises division 17 of the same system onwards (figure 2.2).

The tissue surrounding the airway and the alveolus is assumed to produce nitric oxide at a constant rate and that this can either:

- Diffuse into the blood, which acts as an infinite sink

- Diffuse into the airway

- Be consumed by reactions with substances within the cell (superoxide, metalloproteins, thiols, oxygen)

Therefore nitric oxide is diffusing into and out of the airway and alveolus at a rate dependent on the level of nitric oxide in the airway, the rate of NO production and NO diffusion into the bloodstream. Using these assumptions it is possible to use the exhaled nitric oxide concentration, rate of NO production and flow rate of exhalation to estimate the alveolar nitric oxide level and a number of other parameters.

Tsoukias and George's method (222) involves plotting the rate of NO production (V_{NO}) against the rate of exhalation and performing linear regression to fit a line to the points obtained. This results in the intercept of the Y-axis providing an estimation of airway nitric oxide flux and the gradient of the derived line providing an estimate of alveolar NO from the equation:

$$V_{NO} = Calv.V_E + J'aw_{NO}$$

Where V_{NO}= elimination rate of exhaled nitric oxide (ml/s), V_E = exhalation flow rate (ml/s), Calv= alveolar NO concentration (ppb), J'aw$_{NO}$=maximum NO flux (pl/sec).
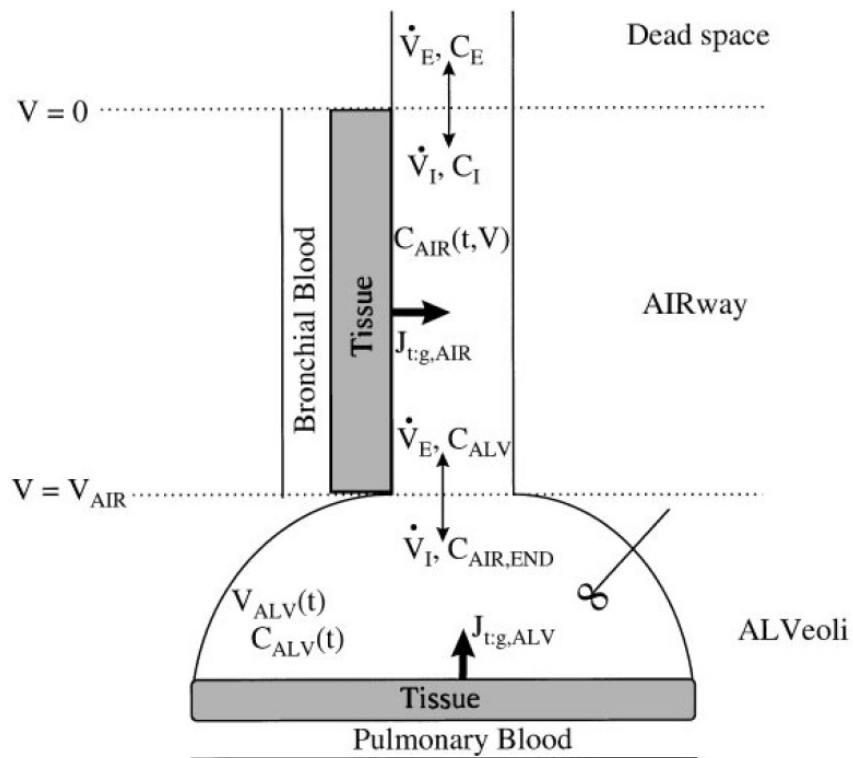
Figure 2.2 Schematic of 2-compartment model for nitric oxide pulmonary exchange.

First compartment represents relatively nonexpansile conducting airways; second compartment represents expansile alveoli. Each compartment is adjacent to a layer of tissue that is capable of producing and consuming nitric oxide (NO). Exterior to tissue is a layer of blood that represents bronchial or pulmonary circulation and serves as an infinite sink for NO. $\dot{V}_E$ and $\dot{V}_I$, expiratory and inspiratory flow, respectively; C_E and C_I, expiratory and inspiratory concentration, respectively; C_{air} and C_{alv}, airway and alveolar concentration, respectively; V_{AIR} and V_{ALV}, airway and alveolar volume, respectively; $J_t{:}g,air$ and $J_t{:}g,alv$, total flux of NO from tissue to air and from alveolar tissue, respectively; t, time; V, volume. Adapted from (227).

Silkoff and colleagues developed a non-linear regression method (255) that correlated well with measurements of exhaled NO at 9 flow rates (4.2, 8.5, 10.3, 17.2, 20.7, 38.2, 75.6, 850 & 1550 ml/sec) and enabled calculation of the parameters above. Solving for the following equation provides estimates for alveolar nitric oxide, nitric oxide flux and airway wall nitric oxide concentration and diffusion:

$$F_{E_{NO}} = Caw_{NO} + (Calv - Caw_{NO})e^{(-DawNO/V_E)}$$

Where $F_{E_{NO}}$=exhaled NO concentration (ppb), Caw_{NO}=airway wall concentration (ppb), Daw_{NO}=diffusion from airway wall to airway (pl/s/ppb), Calv =alveolar NO level (ppb), V_E=flow rate of exhalation (ml/sec)

Nitric oxide flux and airway wall nitric oxide diffusion and concentration can also be obtained by linear regression using the V_{NO} and $F_{E_{NO}}$ results from the 30 and 50ml/sec flow rates (255). V_{NO} is plotted against $F_{E_{NO}}$ and linear regression carried out through the points. Nitric oxide flux can then be obtained from the y-intercept and nitric oxide diffusion from the reciprocal of the slope gradient. Airway wall concentration is obtained from the relationship (255):

$$J'aw_{NO} = Caw_{NO}Daw_{NO}$$

Where $J'aw_{NO}$=maximum NO flux (pl/sec), Caw_{NO}=airway wall concentration (ppb), Daw_{NO}=diffusion from airway wall to airway (pl/s/ppb)

Extended flow analysis has demonstrated elevated levels of alveolar nitric oxide in subjects with severe asthma (compared to subjects with mild asthma) (256, 257) and subjects with COPD (225, 258) although this finding is not consistent (52). Smoking does not appear to reduce alveolar nitric oxide levels in normal subjects (226, 259) and alveolar nitric oxide levels are equivalent in smokers and ex-smokers with COPD (52, 225). This suggests that extended flow analysis may provide useful insights into nitric oxide metabolism in smokers with asthma. Given the uncertainties as to the best method for calculating extended flow nitric oxide parameters and the potential that smokers with asthma may have alterations in some or all extended flow nitric oxide parameters both linear and non-linear models were employed. The inherent variability of the presented parameters was not determined in this thesis.

2.5.5 Exhaled breath condensate pH

Exhaled breath condensate (EBC) collection was performed using a Jaeger EcoScreen® (VIASYS GmbH, Leibnizstrasse 7, D-97204 Hoechberg, Germany) which complied with expert opinion (56). Subjects performed tidal breathing into the apparatus mouthpiece for a minimum of ten minutes whilst wearing a nose-clip. Subjects were allowed brief rests if required and were instructed to cease breathing into the mouthpiece prior to removal of nose-clip and to avoid expelling flatus into the mouthpiece. If an insufficient amount of EBC was collected after ten minutes the subjects were asked to continue for a further 5 minutes. A final further five minutes were allowed if there was still insufficient fluid after fifteen minutes.

The EBC sample obtained was processed as per expert opinion (56, 232). One ml of the sample was aliquoted into a 1.5ml eppendorf and de-aerated for ten minutes with argon resulting in the removal of carbon dioxide from the solution and stabilisation of pH. Recordings of pH were taken using a MINITRODE P electrode (VWR International, Lutterworth, UK) and HANNAH pH 210 digital meter (VWR International, Lutterworth, UK) which had been calibrated prior to use using pH 4 and 7 buffers. Reproducibility of EBC pH measurement was not addressed in this thesis.

2.5.6 Sputum induction

Sputum was obtained using a procedure modified from that of Pin et al (184) and Pavord et al (181). Subjects performed spirometry and then were pre-treated with nebulised salbutamol followed by spirometry after thirty minutes. The highest post salbutamol FEV_1 obtained was recorded for post saline evaluation. Subjects inhaled nebulised saline using an ultrasonic nebuliser (Sonix 2000, Medix Ltd, Harlow, Essex, UK) for three seven minute periods resulting in a maximum of 21 minutes of nebulisation. Hypertonic saline at concentrations of 3, 4 and 5% (Western Infirmary & Gartnavel General Hospital, Sterile Production Unit) were employed.

To ensure no bronchospasm had occurred during the procedure all subjects performed spirometry after each nebulisation period. If no change in FEV_1 was detected (defined as a drop in FEV_1 of less than 10% from post nebuliser values, an increase in FEV_1 or no change) then the subject continued onto the next concentration of saline. However if a drop of more than 20% from the post nebuliser level was observed the procedure was terminated and the subject was treated with nebulised salbutamol. A drop in FEV_1 of less than 20% and greater than 10% resulted in the subject repeating nebulisation at the same concentration of saline. Subjects were encouraged to expectorate into a sterile container at any time during the procedure. The sterile containers were kept in ice during the procedure and all samples were processed in less than two hours.

2.6 Processing of biological samples

2.6.1 Sputum processing

The sputum obtained was processed using the method of Popov et al (260) with some modifications. Upon arrival in the laboratory the whole sample was decanted, weighed and examined by the technicians. Macroscopic appearances were recorded (quality, obvious salivary contamination etc). The volume of the sample dictated the volume of dithiothreitol (DTT) ('Sputolysin', Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK) diluted in Hanks Balanced Salt Solution (HBSS) (Invitrogen, Paisley, UK) to be added to the sample. For samples less than 5ml, 250µl of 0.1% DTT diluted in 750µl HBSS was added to the sample. Thereafter for every 1ml increase in sample volume a further 50µl was added and the HBSS reduced by 50µl i.e. for samples of 6ml 300µl of 0.1% DTT and 700µl HBSS was added. This increase in DTT concentration continued until the samples were greater than 10ml in volume and then 500µl of DTT and 500µl of HBSS was added to the sample regardless of increase in volume.

Mechanical separation of the sample was then performed for at least ten minutes using a sterile Pasteur pipette to ensure proper separation of the specimen. Once this was achieved the sample was then diluted to 30ml using HBSS and forced through a 70µm cell strainer (VWR International, Lutterworth, UK) into a pre-weighed 50ml sterile tube. The tube was then re-weighed and the volume of filtrate obtained was recorded. A total cell count was then performed using a Neubauer haemocytometer with 20µl of the sample diluted 1:1 in Trypan blue (Sigma-Aldrich, Gillingham, UK). The total number of cells, number of dead and alive and squamous cells were recorded and the total number of viable cells (excluding squamous cells) obtained was then calculated. 500µl of a 1×10^6 concentration of sputum cells was aliquoted off for cytospin slides and the rest of the sample was then centrifuged at 1500 rpm for 10 minutes at 4°C. Four 1ml samples of supernatant were aspirated off and stored for future cytokine analysis and the sample pellet was washed and re-suspended in Roswell Park Memorial Institute medium (RPMI 1640) with 10% Foetal Calf Serum added (RPMI-FCS, SIGMA-ALDRICH Ltd, Gillingham, UK) for measurement of sputum macrophage HDAC activity.

2.6.2 Sputum differential counting

Three cytospin slides were produced, air fixed and stained (Romanowsky staining; Lamb Quick-stain kit, Thermo Fisher Scientific Ltd, UK). The differential cell count was performed by counting 400 non-squamous cells from a representative area of the slide and the total and relative percentage of each cell type recorded. Two technicians examined two of the slides independently and the mean of the counts was used for analysis. The third slide was kept in reserve in case of damage to one of the main slides.

2.6.3 Measurement of HDAC activity in sputum macrophages

HDAC activity was assessed using the Fluor-de-Lys^{TM} HDAC activity kit from BIOMOL (BIOMOL Int, Exeter, UK). The cell suspension obtained at the end of sputum processing was plated out in a six well plate (VWR International, Lutterworth, UK) with three wells being used per patient and 1ml of suspension per well. The plate was incubated at 37˚C with 5% CO_2 supplementation for 1 hour to stimulate adherence of macrophages.

HDAC substrate was then added to two wells at a concentration of 200µM per well. The HDAC inhibitor Trichostatin A (TSA) was also added to one of the wells containing HDAC substrate at a concentration of 1µM. The wells were then labelled TSA + and TSA- depending on which had TSA in addition to substrate. The plate was again incubated for one hour at 37˚C with 5% CO_2 supplementation. After one hour the non-adherent cells were removed and the adherent cells washed with RPMI-FSC and lysed with HDAC lysis buffer. The adherent cells were not inspected or subject to a differential count. The cell lysate was then aspirated into labelled eppendorfs which were stored at -80˚C until development. The remaining well was used for cell counting. HDAC activity of the samples was determined through the addition of Fluor-de-Lys^{TM} developer (BIOMOL Int, Exeter, UK) to 10µL of each of the samples in a white 96 well plate. The plate was covered in tin-foil and placed in the dark at room temperature for 30 minutes. The samples were then excited in a fluorimeter and the emitted light was then recorded. The HDAC activity of the sample was calculated using this result, the result from the blank and control wells and the standard curve.

Correction was performed for the cell count obtained from the third well of the six well plate for each sample. Assay variability testing was not performed.

2.6.4 Measurement of sputum supernatant and plasma cytokines

The sputum supernatant and plasma obtained in the study examining the corticosteroid responses of smokers, ex-smokers and non-smokers with asthma was examined for a number of cytokines and chemokines using a commercially available multiplex immunodetection system (25-plex cytokine assay, Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, UK) and a Luminex 100^{TM} analyser (Luminex Corporation, 12212 Technology Blvd, Austin, Texas, USA). This system enables the detection of multiple cytokines and chemokines of interest in small volumes through the use of antibody labelled microspheres in a solid phase sandwich immunoassay. The microspheres bind to the analyte of interest due to their conjugated antibody and the microspheres enable detection of the concentration of analytes due to their internal dyes which when excited by the lasers incorporated within the Luminex 100^{TM} emit specific wavelengths of radiation. The emitted radiation is detected by the analyser and due to each bead having a different 'signature', the detected radiation is converted to a concentration of cytokine/chemokines for each analyte using results obtained from standard curves. The cytokines detected using this approach were eotaxin, granulocyte/monocyte-colony stimulating factor (GM-CSF), interferon-α (IFN-α), interferon-γ (IFN-γ), interleukin (IL) 1-receptor-antagonist (IL-1RA), IL-1ß, IL-2, IL-2 receptor (IL-2R), IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12 (p40/p70 form), IL-13, IL-15, IL-17, Interferon-inducible Protein of 10 kDa (IP-10 aka CXCL10), Monocyte Chemotactic Protein 1 (MCP-1), Monokine Induced by IFN-γ (MIG aka CXC9), Monocyte Inflammatory Protein 1α (MIP-1α aka CCL3), Monocyte Inflammatory Protein 1ß (MIP-1ß aka CCL4), Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES aka CCL5), and Tumour Necrosis Factor-α (TNF-α).

Validation of the Luminex technique was carried out using a 30-plex cytokine and supplied cytokine standards. Serial dilutions confirmed the linearity of the assay for all cytokines in the working range (figure 2.3-IL-6 result). Addition of the reducing agent DTT at concentrations of 0.05 and 0.00125% had no effect on

antigenicity (figure 2.3-IL-6 result). Spiking of samples also demonstrated good correlation between the spiked concentration and sample concentration as detected by Luminex (r=0.64, p<0.001) (figure 2.4).
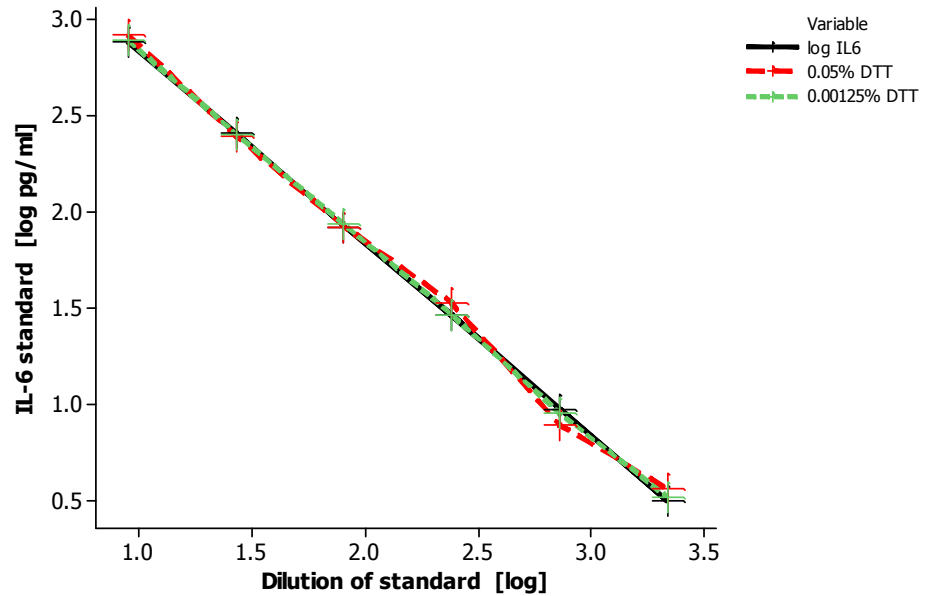
Figure 2.3 Linearity of IL-6 detection and effect of two concentrations of DTT on antigenicity
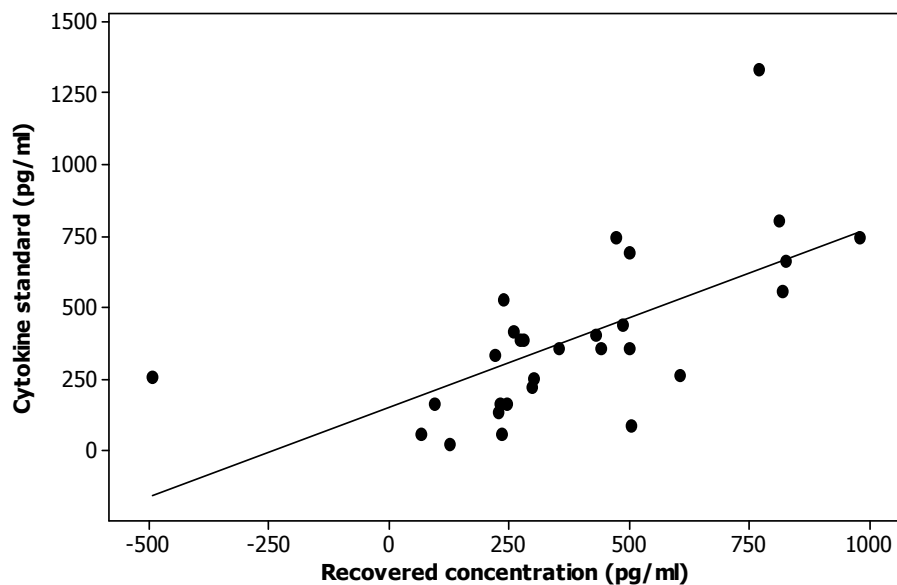
Figure 2.4 Result of spiking experiment for 30 cytokines

In the studies examining theophylline and rosiglitazone in smokers with asthma and the study examining the corticosteroid responses of smokers, ex-smokers and non-smokers with asthma sputum supernatant selected cytokines were also

assessed using pre-coated enzyme linked immunosorbent assays (ELISAs). Sputum supernatant from the study examining theophylline and rosiglitazone was examined for interleukin (IL)-8, myeloperoxidase (MPO) and Regulated on Activation, Normal T Expressed and Secreted (RANTES)/CCL5 levels (IL-8, R&D Systems, Abingdon, UK, MPO, Immundiagnostik, Oxford Biosystems, Oxford, UK, RANTES/CCL5, Invitrogen Ltd, Paisley, UK). Sputum supernatant from the study examining the corticosteroid responses of smokers, ex-smokers and non-smokers with asthma was examined for IL-6 using a high sensitivity ELISA (Abcam plc, Cambridge, UK). Each ELISA plate was processed according to manufacturer's instructions. Briefly the pre-coated plates were loaded with samples followed by incubation with shaking, washing with supplied wash buffer and then streptavidin-horse radish peroxidase was then added to the plates. This was then followed by a further shaking step and a further wash cycle. Developer was then added and the reaction stopped once sufficient colour had developed. The plate was then read on a plate reader with the primary and secondary wavelengths required for the assay and the output determined using the standards within each plate.

2.6.5 Blood tests

2.6.5.1 Peripheral blood monocyte selection

Blood was obtained using lithium heparin vacutainers and the monocyte fraction selected by density centrifugation. Whole blood was diluted 1:1 in RPMI-1640, carefully layered on top of Histopaque® (SIGMA-ALDRICH Ltd, Gillingham, UK) and then centrifuged at 1800rpm for 30 minutes at room temperature. The white cell fraction then became obvious as a creamy white layer at the interface between the Histopaque® and serum. This was carefully aspirated off and washed in RPMI by centrifuging twice for 5 minutes at 1400 rpm at 4˚C. The concentration of cells within the pellet was then determined by cell counting and diluted as appropriate. The pellet obtained was used for the assessment of PBMC HDAC activity.

2.6.5.2 PBMC HDAC activity

HDAC activity in peripheral blood borne monocytes was assessed using the Fluor-de-Lys^{TM} system (BIOMOL Int, Exeter, UK). Briefly the PBMC cell pellet suspension was plated out into three wells of a six well plate and left to adhere for one hour. The non-adherent cells were removed and the HDAC substrate was added to two wells and TSA to one of the two wells. The third well was used for cell counting. After one hour incubation the reaction was stopped using lysis buffer and the resultant lysate scraped off using a cell scraper and aspirated off into three eppendorfs for storage at -80˚C. The HDAC activity of the subjects PBMCs was then assessed by the addition of Fluor-de-Lys developer to the sample. The result was obtained using a fluorimeter as before with correction for cell count. Assay variability testing was not performed.

2.6.5.3 Biochemical assays

Routine biochemistry testing was performed by the North Glasgow Hospital Trust's Biochemistry laboratory, Gartnavel General Hospital, Glasgow. Samples were processed in the study examining theophylline and rosiglitazone for renal function and electrolytes, liver function tests, adjusted calcium, total Cholesterol, glucose and theophylline levels. Serum cortisol concentrations were assessed in the study examining corticosteroid response. All samples were processed and results generated using an automated processing system (ARCHITECT c8000, Abbot Diagnostics, Maidenhead, UK).

2.6.5.4 Differential blood counts

Differential blood counts were performed in the study examining theophylline and rosiglitazone by the North Glasgow and Clyde NHS trust Haematology laboratory at Gartnavel General Hospital, Glasgow using an automated X-Class SYSMEX machine (SYSMEX, Hamburg, Germany). This provided haemoglobin concentration, total and differential white cell count and platelet concentration and a number of other parameters.

2.6.5.5 Serum IgE and IgE antibodies against common allergens

The serum concentrations of IgE and specific immunoglobulin E (IgE) antibody activity against allergens from cat dander, grass pollen and house dust mite were measured by fluorescent enzyme immunoassay (FEIA, UniCAP-100 System, Pharmacia, Milton Keynes, UK) by the North Glasgow and Clyde NHS trust Immunology laboratory, Western Infirmary, Glasgow, in the study examining theophylline and rosiglitazone in smokers with asthma.

The method of FEIA is based on the binding of the patient's serum immunoglobulins to the antigen/s of interest, which is bound to a flexible cellulose matrix, housed within a small permeable container. This is followed by a wash to remove unbound immunoglobulins. IgE bound to the allergen is then detected by binding of beta-galactosidase-labelled anti-IgE, and any unbound secondary antibody is removed by washing. The bound enzyme activity is then measured by catalysing the production of a fluorescent product, umbelliferone, from a colourless substrate. The fluorescence produced is proportional to the IgE antibody concentration, and is quantified by comparison to a standard curve. The assay system has a working range of 0.35-100 arbitrary units per litre (AU/L). A level of greater than 0.35 AU/L for specific IgE is considered raised.

Total serum IgE was measured as above, with the exception that all the serum IgE was captured by anti-IgE bound to the cap matrix. Serum IgE has a log normal distribution, and concentrations greater than 120 IU/L are considered significantly raised, as values above this level are associated with atopy and clinical allergy.

2.7 Data handling and statistical analysis

2.7.1 Data handling

All data for the trial examining theophylline and rosiglitazone was entered into a specially designed case report form (CRF) provided by GlaxoSmithKline (GSK) and sent to their data entry team for generation of the research database. The quality of CRF completion was assessed by an independent monitor employed by GSK who examined ten per cent of the completed CRFs prior to sending for entry

and generated queries for areas which required clarification. Where areas of uncertainty within the data were identified I clarified and corrected where required.

I designed the CRFs and performed all data entry and checking for the study examining corticosteroid responses.

2.7.2 Statistical analysis

2.7.2.1 Approach and performance of analysis

The lung function data in the trial examining theophylline and rosiglitazone was performed by a professional statistician (Dr Lisa Sweeney) employed by GSK using SAS v 8.2 (SAS Institute Inc., NC, USA) on a UNIX system. I performed all other analyses and comparisons in this trial using MINITAB 15 (Minitab Inc. State College, PA, USA). The study analysis was performed with an intention to treat approach.

The majority of the analysis for the trial examining the oral corticosteroid responses of smokers, ex-smokers and non-smokers with asthma was performed by a professional statistician (Dr Chris Weir, Robertson Centre for Biostatistics, University of Glasgow) using SAS v 9.1 (TS1M3) for Windows (SAS Institute Inc., NC, USA). I performed all additional analyses and comparisons using MINITAB 15 (Minitab Inc. State College, PA, USA). This study was analysed as a per-protocol study. Subjects non-compliant with oral corticosteroids formed part of the baseline comparisons but their post oral corticosteroid data was not utilised.

In both studies parametric data was analysed by t–testing and ANOVA as appropriate. Non-parametric data was assessed by Mann Whitney or log transformed prior to normality testing (using Anderson-Darling) and if parametric as a result was analysed by t-testing. Difference of adjusted means analysis (ANCOVA) was performed for the lung function data in the theophylline and rosiglitazone trial due to differences in the baseline characteristics of the groups which were deemed by the statistician to influence the result. All analyses performed for the thesis were two sided with alpha set at 5%.

2.7.2.2 Power calculations

The theophylline and rosiglitazone study did not have a formal power analysis performed due to a lack of information on responses to both drugs in the study population. However the study was informed by the confidence intervals observed in the response of smokers with asthma to an oral corticosteroid trial (23). This resulted in the estimation that 22 subjects were required per group to detect a 230ml difference in FEV_1 between the treatment arms and to allow for a 10% drop-out rate.

The study examining oral corticosteroid responses was informed by a previous trial (23). This resulted in the estimate that 22 subjects were required to provide 80% power to demonstrate a difference of 336ml in FEV_1 response to oral corticosteroids between smokers and non-smokers with asthma and to allow for a 10% drop-out rate.

2.7.2.3 Multiple comparison issues

Both of the studies presented in this thesis are susceptible to false positive results due to multiple comparison issues. These have arisen for several reasons.

In the study examining theophylline and rosiglitazone there is the possibility of a type 1 error despite the use of a pre-defined primary endpoint and alpha level. This is due to the use of four treatment groups and a failure to adjust the alpha level to conserve the family-wise error rate for the trial design. However correction for this issue would have resulted in the study becoming unfeasible for a thesis and for one site to conduct in a reasonable time. The pre-defined secondary endpoints are used to support and confirm the primary endpoint findings in an attempt to reduce erroneous conclusions. Finally a number of exploratory endpoints are permitted for examination of the database to generate future research leads.

The study examining the oral corticosteroid response of smokers, ex-smokers and non-smokers with asthma is again an exploratory study. Given the nature of the study and volume of data generated a number of false positive results are possible given that alpha is set at the traditional value of 5%. However

established methods for correction for multiple analyses were felt to be unhelpful as they are unnecessarily punitive for a study of this size and would obscure potential important observations.

Therefore the conclusions drawn in this thesis are constrained by the likelihood of the presence of false positive results. To compensate, the data is presented as exploratory rather than conclusive. This approach is designed to temper the conclusions and whilst this cannot correct for possible type 1 errors it reminds the reader of the need for confirmation of all findings in adequately powered and informed trials. The final consideration is that adequately powered and conclusive clinical trials in smokers with asthma will now be feasible as a result of the data presented in the following chapters.

3 Reversal of corticosteroid insensitivity in smokers with asthma

3.1 Introduction

Inhaled corticosteroids are recommended as the first-line treatment for chronic persistent asthma (1, 2). However a significant proportion of individuals with asthma fail to establish complete control despite this approach (25).

Smokers with asthma comprise part of this poorly controlled group and exhibit an impaired response to both inhaled and oral corticosteroids compared to non smokers with asthma (5-10, 22-24). Previous research has also demonstrated that smokers with asthma have worse symptoms (11), an accelerated decline in lung function (15, 21) and increased frequency of emergency department visits for asthma (16, 17) compared to matched non-smoking asthmatics.

The prevalence of smoking in asthma reflects that of the general population and therefore smokers with asthma represent a large group of patients with poorly controlled disease (261). Smoking cessation is the obvious route for practitioners and smokers with asthma to pursue and has been demonstrated to be an effective therapy in this group (49), but as sustained quitting rates are low, improvements on current treatments, additional or alternative therapies are required for individuals with asthma who continue to smoke.

What mechanisms are responsible for the reduced response to corticosteroids displayed by smokers with asthma? Corticosteroids reduce inflammation via a number of different mechanisms including inhibition of pro-inflammatory transcription factors through both competition for co-factors and direct inhibition, increased expression of anti-inflammatory genes and repression of inflammatory gene expression (27, 32). One mechanism that corticosteroids use to suppress inflammatory gene expression and that may be of relevance in smokers with asthma has come to light through the research discipline of epigenetics. Epigenetics examines the effect of post-translational covalent modifications of chromatin on the control of gene expression. It has been demonstrated *in-vitro* that approximately half of the immunosuppressant

activity of corticosteroids is produced through stimulating the removal of acetyl groups at areas of active transcription (36). Cigarette smoke reduces HDAC activity *in-vitro* (92), which could explain corticosteroid insensitivity in smokers with asthma. At standard doses, theophylline produces bronchodilation, whereas low doses increase HDAC activity with associated reductions in inflammatory gene expression when given in combination with corticosteroids (102, 103). One of the main aims of this study was to test the hypothesis that low dose theophylline restores HDAC activity in smokers with asthma leading to a restoration of corticosteroid sensitivity which when theophylline was given in combination with inhaled corticosteroid would improve lung function to a greater degree than inhaled corticosteroids alone.

Therefore, I undertook an exploratory clinical trial to examine the effect of low dose theophylline in combination with low dose inhaled corticosteroid on lung function and other outcomes in a group of smokers with asthma.

3.2 Methods

3.2.1 Subjects

Subject characteristics, inclusion/exclusion criteria and recruitment methods were as described in the general methods chapter.

3.2.2 Study design

A full description of the study is provided in the general methods chapter. In brief the study was a randomised, prospective, double-blind, double-dummy, active comparator, parallel group design. Subjects were randomly allocated to one of four treatment groups, three of which are discussed in this chapter. The treatments discussed in this chapter are; twice a day 100mcg inhaled hydrofluoroalkane beclometasone dipropionate alone, the combination of low dose oral theophylline (200mg bd) and inhaled beclometasone dipropionate and theophylline alone (figure 3.1).
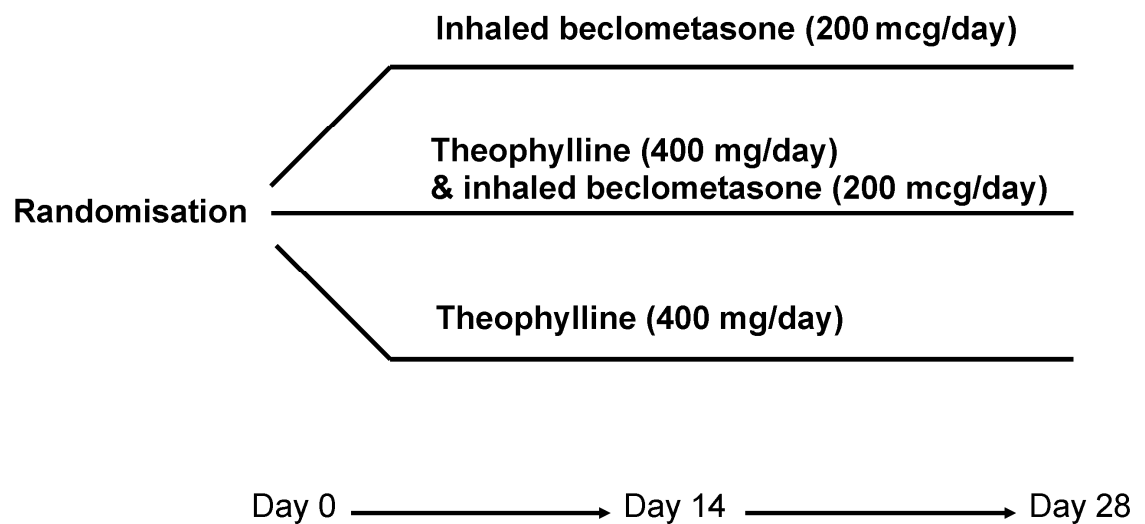
Figure 3.1 Randomisation schedule

Subjects performed a number of assessments at baseline and repeated the assessments after twenty eight days of treatment. A short visit at fourteen days was performed to assess lung function. The West Glasgow Research Ethics Committee approved the study and all patients gave written informed consent.

3.2.3 Measurements

A full description of the measurements is provided in the general methods chapter. Lung function assessments conformed to consensus guidelines (246). Sputum induction, differential count, HDAC measurement and supernatant analysis were performed as discussed in the general methods chapter. HDAC assay variability testing was not performed. Continuation of smoking during the study was confirmed by history and the detection of urinary nicotine metabolites. Subjects were regarded as current smokers if their category was mild smoker or greater and their urine cotinine level was greater than 1.1mg/ml. Treatment compliance was assessed by tablet count, inhaler weight and serum theophylline level.

3.2.4 Statistical analysis

The reduced response to inhaled corticosteroids in smokers with asthma prevented standard power calculations. The study was informed by FEV_1 changes

from a previous clinical trial employing oral corticosteroids in smokers with asthma (23). This resulted in the estimation that 22 subjects were required per group to detect a 230ml difference in FEV_1 between the treatment arms and to allow for a 10% dropout. A slightly higher dropout rate occurred (13%) during the trial resulting in a larger numbers of subjects being randomised to treatment.

The primary endpoint was difference in pre-bronchodilator FEV_1 between the treatments and beclometasone alone at 28 days. The secondary endpoints were change in pre and post bronchodilator PEF, FVC, FEF_{25-75}, FEF_{75} & ACQ. Exploratory endpoints were change in sputum differential, sputum HDAC activity and sputum supernatant. Lung function changes were examined using ANCOVA (incorporating Kenward & Roger's method (262)) using SAS v8.2 (SAS Institute Inc, NC, USA). All data obtained after day 1 of treatment was used for analysis. The remaining statistical analysis was performed using Minitab 15 (Minitab Inc. State College, PA, USA). α was set at 0.05. Due to the exploratory nature of the trial the analyses were not corrected for type 1 errors due to multiple comparisons.

3.3 Results

A total of 3895 subjects with asthma were invited to participate between August 2005 and May 2007, of whom 294 gave positive responses. Following telephone screening, visits were arranged for 187 subjects and 91 subjects met criteria for randomisation (figure 3.2). Sixty-eight subjects were randomised to the portion of the study that is discussed in this chapter. The remaining subjects were randomised to treatment with rosiglitazone and the results for this treatment are discussed in the following chapter. Twenty-three subjects were allocated to the inhaled beclometasone alone and theophylline alone groups and twenty-two to theophylline and inhaled beclometasone. The baseline demographic, clinical (including previous inhaled corticosteroid and long-acting $ß_2$-agonist use) and inflammatory characteristics of recruited subjects in each group were well matched (tables 3.1 & 3.2). All the endpoints presented from this point are the changes seen relative to the inhaled beclometasone group response.
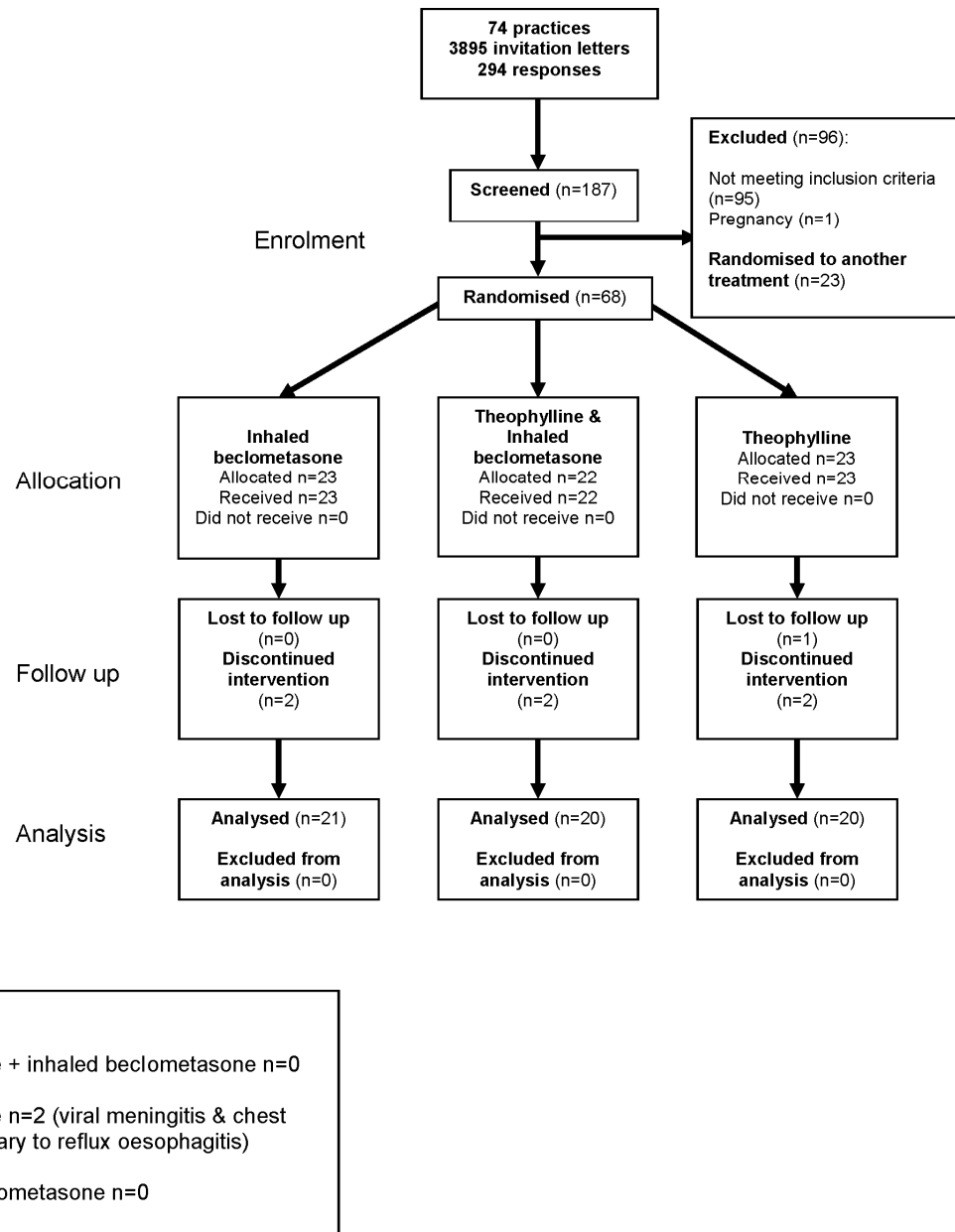
Figure 3.2 CONSORT diagram showing flow of participants through the trial.

SAE; serious adverse event

	Inhaled beclometasone	Theophylline & Inhaled beclometasone	Theophylline
Number of patients	23	22	23
Age (years)	42 (36, 53)	44 (31, 52)	46 (38, 50)
Gender Female (% of total)	61	55	65
BMI (kg/m²) Mean (range)	25.5 (18.4, 34.2)	26.0 (17.3, 36.1)	26.6 (18.6, 37.1)
Pack years	24 (15, 30)	25 (11, 40)	30 (15, 35)
Duration of asthma (years)	16 (8, 31)	15 (9, 21)	16 (9, 30)
Inhaled corticosteroid use at screening (% of total) Dose, beclometasone equivalent (mcg)	65 800 (400, 800)	68 800 (400, 950)	74 400 (400, 900)
LABA use at screening (%)	26	36	35
Specific IgE antibody positive (%)	61	50	52
Total IgE level (IU/ml)	87 (34, 396)	91 (31, 383)	40 (9, 346)
Spirometry (pre-BD) FEV_1 (% predicted)	75 (72, 89)	78 (65, 84)	73 (64, 84)
Reversibility FEV_1 % improvement	16 (13, 20)	15 (14, 18)	18 (14, 24)
Asthma Control Questionnaire score (0 to 6) Mean (SD)	1.8 (0.9)	1.8 (0.7)	2.1 (0.5)

Table 3.1 Baseline demographics.

Data presented as median (IQR) unless stated otherwise. Abbreviations; SD; standard deviation, BMI; Body Mass Index, FEV_1; Forced expiratory volume in 1 second, pre BD; pre-bronchodilator, ACQ; Asthma Control Questionnaire score (range, 0 to 6, with higher scores indicating worse asthma control), IgE; immunoglobulin E, LABA; long-acting β₂-agonist.

	Inhaled beclometasone	Theophylline & Inhaled beclometasone	Theophylline
Sputum total cell count (10^6)	4.3 (2.6, 7.3)	5.1 (3.1, 9.4)	6.0 (2.4, 16.1)
Eosinophils %	0.9 (0.3, 1.6)	0.8 (0.4, 1.8)	1.3 (0.5, 2.3)
Eosinophils (10^4)	2.1 (0.8, 5.8)	3.7 (1.9, 19.5)	6.3 (1.7, 28.4)
Neutrophils %	25.5 (9.6, 44.6)	23.5 (8.6, 42.3)	16.6 (8.4, 40.3)
Neutrophils (10^4)	122.7 (25, 188)	83.0 (35, 302)	80.0 (25, 323)
Macrophages %	52.8 (32.0, 64.4)	45.1 (38.1, 60.8)	52.1 (39.1, 64.3)
Macrophages (10^4)	184.2 (96, 437)	271.8 (165, 452)	78.0 (147, 729)
Lymphocytes %	1.3 (0.6, 2.6)	1.6 (1.0, 2.7)	1.0 (0.5, 2.5)
Lymphocytes (10^4)	4.9 (2.3, 11.2)	12.4 (3.2, 20.7)	7.1 (1.5, 22.6)
Bronchial epithelial cells %	10.5 (8.3, 15.4)	16.4 (8.0, 28.9)	12.3 (6.1, 27.2)
Bronchial epithelial cells (10^4)	40.7 (20.5, 99.4)	119.1 (63.8, 157.6)	83.7 (22.1, 168.2)
HDAC activity AFU/10^6 cells mean (95% CI)	2.25 (0.54, 3.95)	3.75 (0.34, 7.16)	3.62 (0.61, 6.64)

Table 3.2 Baseline sputum counts and HDAC activity.

Data expressed as median (IQR) except where expressed. 95% CI; 95% confidence interval.

3.3.1 Lung function

3.3.1.1 Theophylline and inhaled beclometasone

After two weeks of treatment with low dose theophylline and beclometasone there was a trend for improvement in pre-bronchodilator PEF (24.9 L/min (95% CI –1.5 to 51.2), p=0.064) (figure 3.3) and pre-bronchodilator FVC (132 ml (-23 to 286), p=0.094). There were no detectable differences in other lung function endpoints (table 3.3). After four weeks, treatment with the combination of theophylline and inhaled beclometasone demonstrated a borderline significant improvement in mean pre-bronchodilator FEV_1 (mean difference 165 ml (-13 to 342), p=0.069) (table 3.3 & figure 3.4) and a significant improvement in pre-bronchodilator PEF (39.9 L/min (10.9 to 68.8), p=0.008) (table 3.3 & figure 3.3) and pre-bronchodilator FVC (254 ml (63 to 445), p=0.010) (table 3.3).
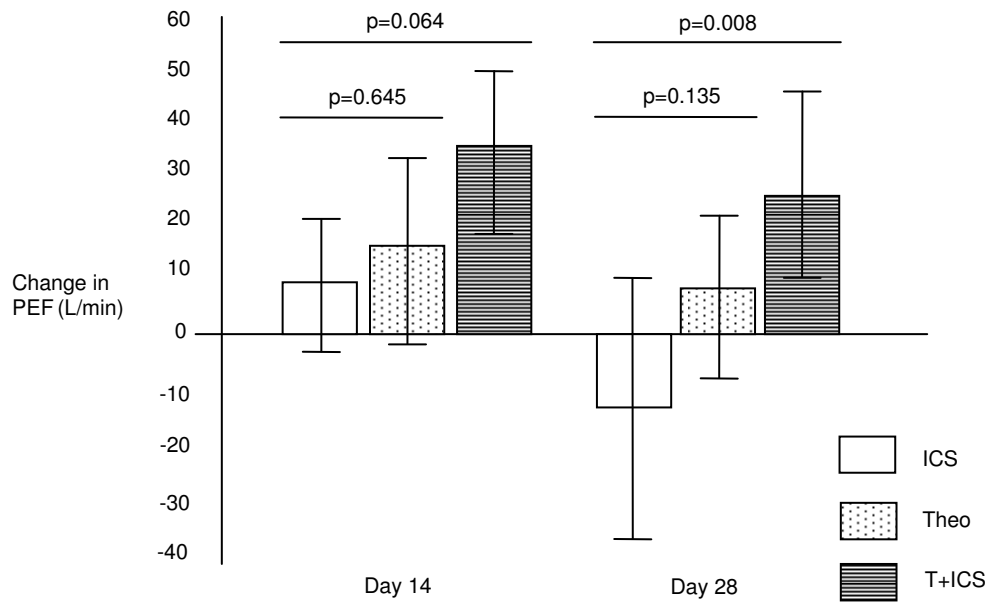
Figure 3.3 Change in PEF (L/min) by 28 days of treatment.

Paired t-test (error bars represent 95% confidence intervals). p-values were derived from comparison of groups to beclometasone dipropionate changes using ANCOVA. Figure key-ICS; inhaled beclometasone, Theo; theophylline, T+ICS; theophylline and inhaled beclometasone combination.
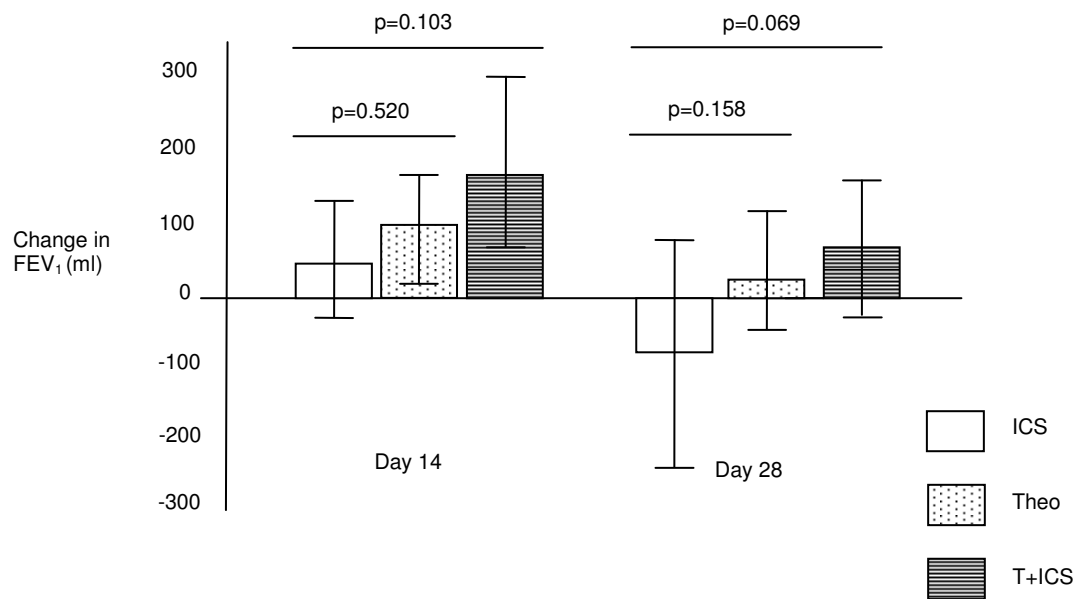
Figure 3.4 Change in FEV$_1$ (ml) by 28 days of treatment.

Paired t-test (error bars represent 95% confidence intervals). p-values were derived from comparison of groups to beclometasone dipropionate changes using ANCOVA. Figure key-ICS; inhaled beclometasone, Theo; theophylline, T+ICS; theophylline and inhaled beclometasone combination

		Theophylline & inhaled beclometasone	Theophylline
Δ Pre BD FEV₁ ml (95% CI)	Day 14	133 (-27, 293)	52 (-109, 214)
	Day 28	165 (-13, 342)	128 (-51, 307)
Δ Pre BD PEF L/min (95% CI)	Day 14	25 (-1, 51)	6 (-20, 33)
	Day 28	40 * (11, 69)	22 (-7, 51)
Δ Pre BD FVC ml (95% CI)	Day 14	132 (-23, 286)	15 (-141, 171)
	Day 28	254 * (63, 445)	176 (-16, 368)
Δ ACQ score (95% CI)		−0.47 * (-0.91, -0.04)	−0.55 * (-0.99, -0.11)
Δ Sputum total cell count	Cells x 10⁶ (95% CI)	-2.0 (-6.3, 1.7)	-1.7 (-6.2, 2.1)
Δ Sputum eosinophil	% (95% CI)	0.0 (-1·1, 0·6)	-0.6 (-1.7, 0.3)
	Absolute (10⁴) (95% CI)	-1.62 (-9·58, 1·82)	-5.53 (-17.87, 1.68)
Δ Sputum neutrophil	% (95% CI)	0.3 (-12.3, 17.7)	-2.5 (-22.5, 12.8)
	Absolute (10⁴) (95% CI)	46.8 (-65.1, 236.2)	-16.0 (-199.5, 116.1)
Δ Sputum macrophage	% (95% CI)	0.5 (-11.8, 11.3)	-2.5 (-21.0, 15.3)
	Absolute (10⁴) (95% CI)	-52.7 (-251.4, 118.7)	-0.9 (-250.0, 186.3)
Δ Sputum lymphocyte	% (95% CI)	-0.8 * (-1.4, -0.1)	-0.6 (-1.3, 0.2)
	Absolute (10⁴) (95% CI)	-10.99 * (-18.15, -1.65)	-3.98 (-10.30, 1.36)
Δ Sputum bronchial epithelial cell	% (95% CI)	1.2 (-5.8, 7.4)	-1.0 (-11.3, 5.7)
	Absolute (10⁴) (95% CI)	-20.9 (-85.1, 50.5)	-12.9 (-100·8, 57·3)
Δ Sputum IL-8	pg/ml (95% CI)	-562.5 (-2131.0, 131.4)	-1201.3 * (-2409.6, -76.6)
Δ Sputum MPO	ng/ml (95% CI)	-126.6 (-433.9, 58.1)	-215.0 * (-556.0, -36.7)
Δ HDAC activity	AFU/10⁶ cells (95% CI)	-3.5 (-23.7, 5.0)	-2.2 (-23.4, 3.5)

Table 3.3 Change in lung function and biomarkers following treatment (relative to response to treatment with inhaled beclometasone alone).

*; p<0.05

3.3.1.2 Theophylline

The group treated with theophylline alone did not demonstrate efficacy for any lung function outcome except for post-bronchodilator FVC at four weeks (304 ml (95% CI 5 to 604), p=0.046).

3.3.1.3 ACQ score

After four weeks, the combination of theophylline and inhaled beclometasone produced a significant improvement in ACQ score (-0.47 (95% CI –0.91 to –0.04), p=0.033) (figure 3.5 and table 3.3). Theophylline alone also reduced the ACQ score (-0.55 (–0.99 to –0.11), p=0.016).
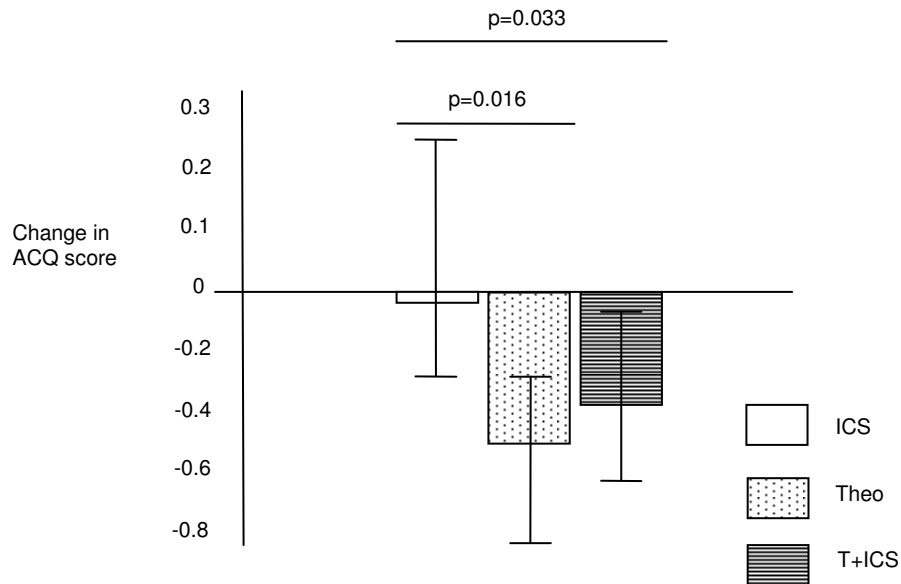
Figure 3.5 Change in ACQ score by 28 days of treatment.

Paired t-test (error bars represent 95% confidence intervals). p-values were derived from comparison of groups to beclometasone dipropionate changes using ANCOVA. Figure key-ICS; inhaled beclometasone, Theo; theophylline, T+ICS; theophylline and inhaled beclometasone combination

3.3.2 Sputum samples

3.3.2.1 Induced sputum cytology

Ninety-seven percent of the subjects who completed the trial produced a sample adequate for analysis both pre and post-treatment. Treatment with the

combination of theophylline and inhaled beclometasone was associated with a reduction in the mean absolute (-10.99 (95% CI -18.15, -1.65), p=0.018) and percentage sputum lymphocyte count (-0.8% (-1.4, -0.1), p=0.028) (table 3.3). No other relative treatment differences in sputum proportions were observed.

Given the change in sputum lymphocytes, sputum supernatant RANTES levels were subsequently examined. However no difference was detected in RANTES expression following treatment with theophylline and inhaled beclometasone (-0.131pg/ml (-0.849, 0.528), p=0.487).

3.3.2.2 Inflammatory biomarkers in sputum

At four weeks, treatment with theophylline alone was associated with a reduction in sputum supernatant IL-8 (-1201.3 pg/ml (95% CI, -2409.6, -276.6), p=0.009) and MPO (-215.0 ng/ml (-556.0, -36.7), p=0.026) measured by ELISA (table 3.3). No significant changes were detected in sputum IL-8 or MPO in the group treated with theophylline and inhaled beclometasone.

3.3.2.3 HDAC activity

HDAC activity was measurable for a subgroup within each treatment group [inhaled beclometasone n=4, theophylline alone n=7, theophylline and inhaled beclometasone n=7]. The majority of samples had a low level of HDAC activity. No difference was obvious between the groups at baseline or after treatment (Tables 3.2 & 3.3 and figure 3.6).

3.3.3 Serum theophylline levels

Serum concentrations were below the current recommended target range (10-20µg/ml). The mean serum concentration for the theophylline alone group (4.9µg/ml, SD 2.4) was similar to that achieved in the theophylline and inhaled beclometasone group (4.3µg/ml, SD 2.0).
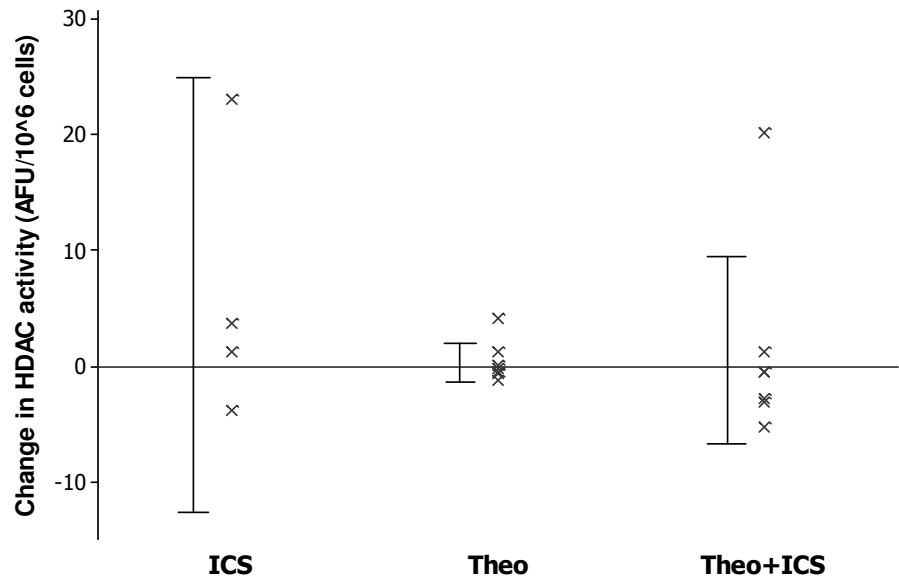
Figure 3.6 Change in HDAC activity from randomisation to 28 days of treatment.

Individual plots of HDAC activity shown. Error bars represent 95% confidence intervals. Figure key- ICS; inhaled beclometasone, Theo; theophylline, Theo + ICS; theophylline and inhaled beclometasone combination

3.3.4 Compliance

Eighty seven percent of the subjects who completed the trial achieved greater than 80% compliance with therapy.

3.3.5 Adverse events

Two serious adverse events occurred during the trial. Both occurred in the theophylline alone arm. One subject was admitted with viral meningitis and another with chest pain due to gastro-oesophageal reflux (a pre-existing condition). Neither subject withdrew from the study. There were two withdrawals due to adverse events. One each occurred in the inhaled beclometasone alone (diarrhoea and vomiting) and theophylline and inhaled beclometasone (headache) arms. The frequency of headache was equal between the groups (six reported for theophylline and inhaled corticosteroids, seven for theophylline and five for low dose beclometasone). Gastrointestinal upset was common in the theophylline alone group with fourteen episodes being reported. Two subjects reported nausea whilst on theophylline and low dose beclometasone but no other gastrointestinal symptoms were reported.

Pharyngitis was reported by three subjects in the low dose beclometasone alone group.

3.4 Discussion

Previous research has demonstrated that the therapeutic response to inhaled corticosteroids is impaired in smokers with asthma (7,, 8-10), highlighting the need for alternative treatment approaches for this large subgroup of asthma. This exploratory clinical trial examined the efficacy of an alternative approach to treatment of smokers with mild to moderate asthma with the aim of restoring corticosteroid sensitivity. The hypothesis behind the study was based on previous research that suggested that the addition of low dose theophylline to an inhaled corticosteroid would improve lung function to a greater degree than inhaled corticosteroid alone due to a restoration of HDAC activity. This study shows that the combination of theophylline and inhaled corticosteroid produces improvements in several indices of lung function and improves asthma control in smokers with asthma.

Treatment with the combination of oral theophylline and inhaled beclometasone was associated with a borderline significant increase in pre-bronchodilator FEV_1 after 28 days of treatment and with a large improvement in both pre-bronchodilator PEF and FVC. The improvement seen in both PEF and FVC (and the associated drop in ACQ score) make it likely that the observed improvement in FEV_1 is real and not a result of multiple comparisons. Given its exploratory nature and hence small numbers of participants it is likely that the study was slightly underpowered to conclusively demonstrate the change in FEV_1 with treatment. A post hoc power calculation, based on the between patient standard deviation (285 ml) for the theophylline and inhaled corticosteroid group suggests that 48 subjects per group are required for 80% power to detect a FEV_1 difference of 165ml between the combination of theophylline and inhaled corticosteroid and inhaled corticosteroid alone arms. The power calculation for PEF based on the between patient standard deviation (46.4 L/min) for the theophylline and inhaled corticosteroid group, reveals that 23 subjects per group would be required to provide 80% power for the detection of a 40 L/min difference in PEF.

The size of the improvement seen following treatment with low dose theophylline and inhaled beclometasone (40 L/min) is likely to be clinically significant as it is larger (263, 264) or equivalent (265) to the improvements seen when long acting β_2-agonists are added to inhaled corticosteroids in non-smoking asthmatics. Furthermore, this improvement is much larger than that produced by montelukast (9) and high dose inhaled corticosteroids in smokers with asthma (8). The improvements in lung function following treatment with low dose theophylline and inhaled beclometasone were also associated with a reduction in ACQ score that was just below the clinically significant threshold of 0.5 (254). Therefore further research, powered on these findings, should be carried out using low dose theophylline and inhaled corticosteroid to confirm and extend our understanding of the efficacy of this combination in smokers with asthma.

Theophylline alone did not produce any significant changes in pre-bronchodilator lung function relative to that produced by inhaled beclometasone. Nevertheless low dose theophylline treatment did produce an increase in post-bronchodilator FVC, a clinically significant reduction in ACQ score and reduction in sputum supernatant cytokines. Previous research has demonstrated that theophylline has this effect in COPD (266). No direct comparison was made between low dose theophylline and the combination of low dose theophylline and inhaled corticosteroid. However, it would appear that if this comparison was made, there would be no clear difference between the two arms. Therefore low dose theophylline alone may provide an alternative therapy in smokers with asthma as it resulted in a clear improvement in asthma symptoms (as measured by the asthma control questionnaire) and could also produce a clear improvement in lung function relative to inhaled corticosteroid in an adequately powered trial (albeit smaller than that seen with the combination of low dose theophylline and inhaled corticosteroid).

Theophylline has many modes of action including non-specific phosphodiesterase activity and adenosine receptor antagonism (100) and both of these mechanisms could produce bronchodilation. However the improvement in lung function with low dose oral theophylline in combination with inhaled beclometasone is unlikely to be due to a bronchodilating effect of theophylline alone, given the absence of statistically significant improvements in lung function with low dose theophylline

alone. Therefore there appears to be a synergistic interaction between low dose theophylline and beclometasone.

Given the many suggested mechanisms of theophylline there are several ways by which low dose theophylline could synergise with corticosteroids to improve lung function in smokers with asthma. One potential mechanism that I have attempted to address is the ability of low dose theophylline to restore HDAC activity. Exposure to cigarette smoke *in-vitro* can reduce HDAC activity and this can be restored by low doses of theophylline (92, 102) leading to the hypothesis that reduced HDAC activity is responsible for the reduced corticosteroid response seen in smokers with asthma. The serum concentration of theophylline achieved in the subjects was within the range previously demonstrated to stimulate HDAC activity. Unfortunately an increase in HDAC activity was not observed in those subjects treated with theophylline and inhaled beclometasone. The reason for this may be explained by the low number of sputum macrophages harvested for analysis. The subjects recruited were able to produce specimens of sufficient quality for differential counting and supernatant analysis, but the number of macrophages harvested for HDAC was low and at the detection limit of the technique. Another consideration is that there was no step to allow for inspection of the cells selected for the HDAC assay. Therefore there is the possibility that contamination with non-viable cells and neutrophils may have affected the results and resulted in the observed low levels of HDAC activity. Future work examining theophylline in smokers with asthma needs to address the underlying mechanism/s responsible. Bronchoalveolar lavage samples should be obtained to allow sufficient macrophages for HDAC analysis and to ensure comparisons can be made with previous research. As previously mentioned theophylline can also act as a non-specific phosphodiesterase inhibitor and an adenosine receptor antagonist and the contribution of these (and other) mechanisms to synergism between theophylline and inhaled beclometasone should be examined in smokers with asthma.

The present study has also demonstrated that treatment with theophylline and inhaled beclometasone is associated with a reduction in sputum lymphocytes. How a reduction in the number of airway lymphocytes following the addition of low dose theophylline would lead to an improved response to inhaled beclometasone is unclear at present. A possible explanation is that the reduction

observed is spurious due to the group treated with theophylline and inhaled beclometasone having a slightly higher sputum lymphocyte count at baseline (albeit non-significantly different). Therefore a small drop in the sputum lymphocytes in this group combined with a small increase in the inhaled beclometasone treated group could produce this apparent drop. Previous work addressing the reproducibility of induced sputum counts has also demonstrated that sputum lymphocyte counts display lower reproducibility compared to eosinophils and neutrophils so we may be observing the inherent variability of this aspect of induced sputum (267). However low dose theophylline has previously been demonstrated to reduce bronchoalveolar lavage lymphocyte numbers with an associated alteration in the CD4/CD8 ratio and reduction in the late asthmatic response to allergen challenge (268). Therefore the observed reduction in sputum lymphocytes may reflect a true effect of theophylline in this patient group. If this issue is to be addressed, in future studies examination of bronchial biopsy samples and bronchoalveolar lavage samples will be required. This approach would allow identification of lymphocyte sub-types and their response to low dose theophylline and inhaled corticosteroid therapy. The absence of other changes in sputum inflammatory cell profiles is likely to be due to both the short duration of treatment and the absence of sputum eosinophilia and neutrophilia in the patients studied.

3.5 Conclusions

This pilot study demonstrates improvements in both lung function and asthma control from the addition of low dose theophylline to inhaled beclometasone in a group of smokers with mild to moderate asthma. The presented results are encouraging given the documented poor response of smokers with asthma to standard doses of inhaled corticosteroids and the need for more effective therapies in this group. Important questions that need to be addressed in future trials include:

- the effect of lower doses of theophylline and hence the lowest effective dose

- the relative performance of the combination of low dose theophylline and low dose inhaled corticosteroid to

    o high dose inhaled corticosteroid

    o combined long acting beta agonist and inhaled corticosteroid

    o leukotriene receptor antagonists

4 Efficacy of a PPARγ agonist in a group of smokers with asthma

4.1 Introduction

Inhaled corticosteroids are recommended as the first-line treatment for chronic persistent asthma (1, 2). However a significant proportion of individuals with asthma fail to establish complete control with this approach (25). Despite the use of additional therapies (including oral corticosteroids) around 10% of subjects with asthma have poorly controlled symptoms and this group are estimated to consume 50% of the costs associated with the treatment of asthma (29, 32). Few new treatments have become available for asthma since the introduction of inhaled corticosteroids with the exception of leukotriene receptor antagonists and anti-IgE therapy, both of which offer some improvements in control but are generally regarded as inferior to corticosteroids. A number of targeted treatment approaches are in development, for example p38 MAPK inhibitors, with the hope that these will provide better control of corticosteroid resistant disease. However the beneficial effects of corticosteroids depend on their ability to act simultaneously via a number of mechanisms and pathways. Therefore the narrow focus of targeted therapies could mean that they will only be able to provide improvements in a small proportion of patients.

Smokers with asthma exhibit an impaired response to both inhaled and oral corticosteroids (5-10, 22-24), an accelerated decline in lung function (15, 21), increased emergency department visits for asthma (with associated costs) (16, 17) and increased severity of symptoms compared to non-smoking asthmatics (11, 12). The prevalence of smoking in asthma reflects that of the general population and therefore smokers with asthma comprise a large group of patients with poorly controlled disease (261). Smoking cessation is an effective therapy in this group (49), but as sustained quitting rates are low, additional or alternative therapies are needed for individuals with asthma who continue to smoke.

The glucocorticoid receptor is a member of the nuclear hormone receptor family, which includes peroxisome proliferator activated receptor-γ (PPARγ). PPAR-γ agonists exert anti-inflammatory effects on multiple inflammatory cell subtypes *in-vitro* and reduce inflammation in animal models of both asthma and neutrophilic airways disease (113, 114). Based on this evidence and the hypothesis that the PPAR-γ agonist rosiglitazone would have beneficial anti-inflammatory actions in smokers with asthma I undertook an exploratory clinical trial to examine the effect of rosiglitazone on lung function and other outcomes in a group of smokers with asthma.

4.2 Methods

4.2.1 Subjects

Subject characteristics, inclusion/exclusion criteria and recruitment methods were as described in the general methods chapter. All subjects provided informed consent and the study was approved by the West Glasgow Ethics Committee.

4.2.2 Study design

A full description of the study is provided in the general methods chapter. In brief the study was a randomised, prospective, double-blind, double-dummy, active comparator, parallel group design. Subjects were randomly allocated to one of four treatment groups, two of which are discussed in this chapter. Subjects were randomised to either 4 mg twice a day oral rosiglitazone maleate or 100mcg twice a day inhaled hydrofluoroalkane beclometasone dipropionate (figure 4.1). Subjects returned for pre-bronchodilator lung function at two weeks and repeated the assessments carried out at the baseline visit after four weeks.
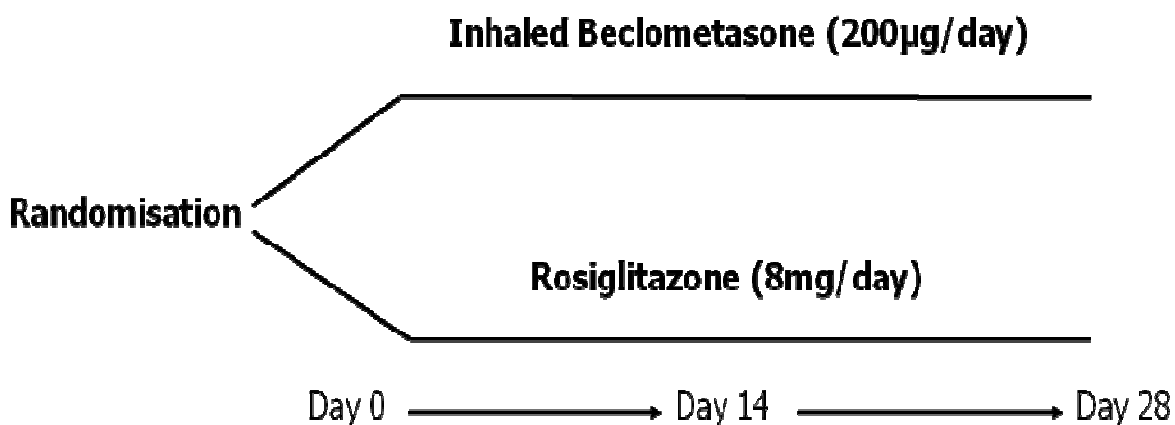
Figure 4.1 Randomisation schedule

4.2.3 Measurements

A full description of the measurements is provided in the general methods chapter. Lung function assessments conformed to consensus guidelines (246). Sputum induction, differential count and supernatant analysis were performed as discussed in the general methods chapter. Continuation of smoking during the study was confirmed by history and the detection of urinary nicotine metabolites. Subjects were regarded as current smokers if their category was mild smoker or greater and their urine cotinine level was greater than 1.1mg/ml. Treatment compliance was assessed by tablet count and inhaler weight.

4.2.4 Statistical analysis

The reduced response to inhaled corticosteroids in smokers with asthma combined with a lack of published information on the effect of thiazolidinediones in asthma prevented the performance of standard power calculations. The study was informed by FEV_1 changes from a previous clinical trial employing oral corticosteroids in smokers with asthma (23). This resulted in the estimate that 22 subjects were required per group to detect a 230ml difference in FEV_1 between the treatment arms and to allow for a 10% dropout rate. A slightly higher dropout rate occurred (13%) during the trial resulting in a short extension to allow a larger numbers of subjects to be randomised to treatment.

The primary endpoint was difference in pre-bronchodilator FEV_1 between rosiglitazone and beclometasone alone at 28 days. The secondary endpoints were change in pre and post bronchodilator PEF, FVC, FEF_{25-75}, FEF_{75} & ACQ. Exploratory endpoints were change in sputum differential & supernatant and serum cytokines. Lung function changes were examined using ANCOVA (incorporating Kenward & Roger's method (262)) using SAS v8.2 (SAS Institute Inc, NC, USA). All data obtained after day one of treatment was used for analysis. The remaining statistical analysis was performed using Minitab 15 (Minitab Inc. State College, PA, USA). α was set at 0.05. Due to the exploratory nature of the trial the analyses were not corrected for type 1 errors due to multiple comparisons.

4.3 Results

A total of 3895 subjects with asthma were invited to participate between August 2005 and May 2007, of whom 294 gave positive responses. Following telephone screening, visits were arranged for 187 subjects and 91 subjects met criteria for randomisation (Figure 4.2). Forty five subjects were randomised to theophylline and theophylline and inhaled beclometasone. The results from the forty-six subjects randomised to either rosiglitazone or inhaled beclometasone will be discussed from this point. Twenty-three subjects were allocated to rosiglitazone and twenty-three to inhaled beclometasone alone. The baseline demographic, clinical (including previous inhaled corticosteroid and long-acting β_2-agonist use) and inflammatory characteristics of recruited subjects in each group were well matched (tables 4.1 & 4.2). All the endpoints presented are the changes relative to the inhaled corticosteroid group response.

74 practices
3895 invitation letters
294 responses

Screened (n=187)

Excluded (n=96):
Not meeting inclusion criteria (n=95)
Pregnancy (n=1)
Randomised to other treatments
(n=45)

Enrolment

Randomised (n=46)

Rosiglitazone
Allocated n=23
Received n=23
Did not receive n=0

Inhaled beclometasone
Allocated n=23
Received n=23
Did not receive n=0

Allocation

Lost to follow up
(n=2)
Discontinued intervention
(n=3)

Lost to follow up
(n=0)
Discontinued intervention
(n=2)

Follow up

Analysed (n=18)

Excluded from analysis (n=0)

Analysed (n=21)

Excluded from analysis (n=0)

Analysis

SAE

Rosiglitazone n=0

Inhaled steroid n=0

Figure 4.2 CONSORT diagram.
SAE; serious adverse event.

	Inhaled beclometasone	Rosiglitazone
No of patients	23	23
Age (years)	42 (36, 53)	41 (33, 54)
Gender Female (% of total)	61	57
BMI (kg/m²) Mean (range)	25.5 (18.4, 34.2)	26.1 (19.5, 38.6)
Pack years	24 (15, 30)	21 (13, 40)
Duration of asthma (years)	16 (8, 31)	18 (6, 29)
Inhaled corticosteroid use at screening (% of subjects)	65	83
Dose, beclometasone equivalent mcg	800 (400, 800)	800 (400, 800)
LABA use at screening (%)	26	30
Specific IgE antibody positive (%)	61	78
Total IgE level (IU/ml)	87 (34, 396)	239 (49, 488)
Spirometry Pre-BD FEV_1 (% predicted)	75 (72, 89)	70 (60, 89)
Reversibility FEV_1 % improvement	16 (13, 20)	16 (13, 26)
Asthma Control Questionnaire score (0 to 6) Mean (SD)	1.8 (0.9)	1.9 (0.7)

Table 4.1 Baseline demographics.

Data presented as median (interquartile range) unless stated otherwise. Abbreviations; SD; standard deviation, BMI; Body Mass Index, FEV_1; Forced expiratory volume in 1 second, pre BD; pre bronchodilator, ACQ; Asthma Control Questionnaire score (range, 0 to 6, with higher scores indicating worse asthma control), IgE; immunoglobulin E, LABA; long-acting β_2-agonist., mcg; microgram, IU/ml; international units per ml, kg/m² ; kilograms per square metre.

	Inhaled beclometasone	Rosiglitazone
Sputum total cell count (10^6)	4.3 (2.6, 7.3)	4.7 (2.4, 9.9)
Eosinophils %	0.9 (0.3, 1.6)	1.1 (0.5, 3.0)
Eosinophils (10^4)	2.1 (0.8, 5.8)	5.1 (1.5, 17.9)
Neutrophils %	25.5 (9.6, 44.6)	28.8 (13.1, 46.2)
Neutrophils (10^4)	122.7 (25, 188)	150.3 (27, 492)
Macrophages %	52.8 (32.0, 64.4)	48.0 (26.1, 64.1)
Macrophages (10^4)	184.2 (96, 437)	185.8 (105, 355)
Lymphocytes %	1.3 (0.6, 2.6)	1.4 (0.7, 2.0)
Lymphocytes (10^4)	4.9 (2.3, 11.2)	8.0 (2.1, 13.5)
Bronchial epithelial cells %	10.5 (8.3, 15.4)	11.0 (6.7, 18.8)
Bronchial epithelial cells (10^4)	40.7 (20.5, 99.4)	49.6 (28.4, 110.6)

Table 4.2 Baseline sputum counts.

Data expressed as median (IQR)

4.3.1 Lung function

At two weeks, rosiglitazone demonstrated a borderline improvement in pre-bronchodilator FEV_1 (164 ml, (95% CI –1 to 329), p=0.051), a significant improvement in pre-bronchodilator PEF (32.7 L/min, (5.7 to 59.7), p=0.018) and significant improvement in both FEF_{25-75} (0.36 L/sec, (0.088 to 0.632), p=0.010) and FEF_{75} (0.24 L/sec, (0.094 to 0.386), p=0.002)(table 4.3). After four weeks, the group treated with rosiglitazone demonstrated a borderline improvement in pre-bronchodilator FEV_1 (183ml (–1 to 367), p=0.051) (figure 4.3) and a significant improvement in FEF_{25-75} (0.243 L/sec (0.025 to 0.461) p=0.030) (figure 4.4 and table 4.3). There was no difference between the groups treated with rosiglitazone and inhaled beclometasone for other measurements of lung function.

4.3.2 ACQ score

There was no difference between the rosiglitazone and inhaled beclometasone groups when changes in ACQ scores were compared (table 4.3).

		Rosiglitazone
Δ Pre BD FEV$_1$	Day 14	164 [†] (-1, 329)
ml (95% CI)	Day 28	183 [†] (-1, 367)
Δ Pre BD FVC	Day 14	45 (-114, 204)
ml (95% CI)	Day 28	156 (-42, 354)
Δ Pre BD PEF	Day 14	33 [*] (5, 59)
L/min (95% CI)	Day 28	23 (-6, 53)
Δ Pre BD FEF$_{25\text{-}75}$	Day 14	0.360 [*] (0.088, 0.632)
L/sec (95% CI)	Day 28	0.243 [*] (0.025, 0.461)
Δ Pre BD FEF$_{75}$ **L/sec (95% CI)**	Day 14	0.240 [*] (0.094, 0.386)
	Day 28	0.111 (-0.011, 0.233)
Δ ACQ score	Δ (95% CI)	-0.07 (-0.52, 0.38)

Table 4.3 Change in lung function and ACQ following treatment (relative to response to inhaled beclometasone alone).

PEF; peak expiratory flow, FVC; forced vital capacity, FEF$_{25\text{-}75}$; forced mid-expiratory flow rate, FEF$_{75}$; forced expiratory flow at 75% of FVC, 95% CI; 95 percent confidence intervals. *; p<0.05, †; p=0.05
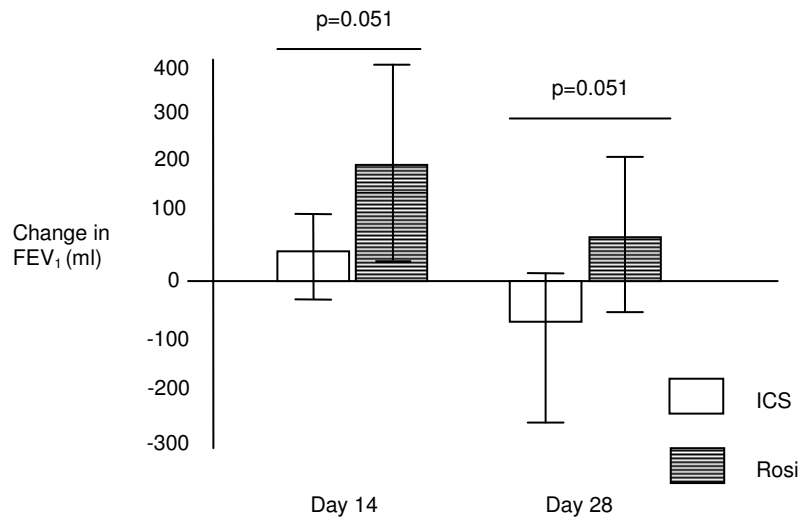
Figure 4.3 Mean group FEV$_1$ changes from randomisation to 14 and 28 days of treatment

Paired t-test (error bars represent 95% confidence intervals)). p-values were derived from comparison of rosiglitazone group change to inhaled beclometasone dipropionate change using ANCOVA.
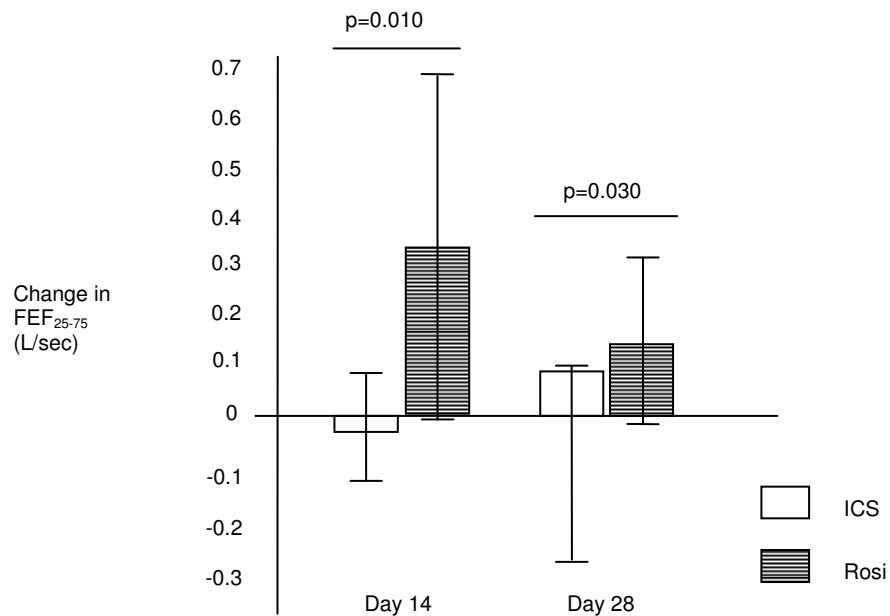
Figure 4.4 Mean group FEF$_{25-75}$ changes from randomisation to 14 and 28 days of treatment

Paired t-test (error bars represent 95% confidence intervals). p-values were derived from comparison of rosiglitazone group change to inhaled beclometasone dipropionate change using ANCOVA.

4.3.3 Sputum samples

4.3.3.1 Induced sputum cytology

No relative treatment differences in sputum proportions were observed (table 4.4).

4.3.3.2 Sputum supernatant

A borderline relative reduction in sputum IL-8 was observed in the group treated with rosiglitazone (-534.1pg/ml, (95% CI -1844.4, 36.5), p=0.068) (table 4.4).

4.3.4 Compliance

Eighty-five percent of the subjects who completed the study achieved greater than 80% compliance with therapy.

4.3.5 Adverse events

No serious adverse events occurred in the rosiglitazone and inhaled beclometasone arms during the trial. There were two withdrawals due to adverse events. One each occurred in the rosiglitazone (periorbital oedema) and inhaled beclometasone alone (diarrhoea and vomiting) arms. The frequency of headache was equal between the groups (five for low dose beclometasone and four for rosiglitazone). Three subjects in the low dose beclometasone alone group reported pharyngitis.

		Rosiglitazone
Δ **Sputum total cell count**	Δ (10^6) (95% CI)	1.3 (-2.1, 4.7)
Δ **Sputum eosinophil**	**%** (95% CI)	0.1 (-1.0, 1.3)
	Absolute (10^4) (95% CI)	1.42 (-4.7, 6.4)
Δ **Sputum neutrophil**	% (95% CI)	4.5 (-16.5, 26.5)
	Absolute (10^4) (95% CI)	32.9 (-120.4, 201.1)
Δ **Sputum macrophage**	% (95% CI)	3.4 (-13.3, 16.8)
	Absolute (10^4) (95% CI)	110.0 (-27.2, 326.8)
Δ **Sputum lymphocyte**	% (95% CI)	-0.5 (-1.1, 0.4)
	Absolute (10^4) (95% CI)	-0.19 (-7.0, 5.2)
Δ **Sputum bronchial epithelial cell**	% (95% CI)	-4.7 (-11.3, 2.0)
	Absolute (10^4) (95% CI)	5.9 (-65.8, 78.4)
Δ **Sputum IL-8**	pg/ml (95% CI)	-534.1 (-1844.4, 36.5)
Δ **Sputum MPO**	ng/ml (95% CI)	-91.3 (-335.1, 44.2)

Table 4.4 Change in sputum counts and supernatant cytokines following treatment (relative to treatment with inhaled beclometasone alone).

Δ; change in endpoint, IL-8; interleukin-8, MPO; myeloperoxidase, pg/ml; picogrammes per millilitre, ng/ml; nanogrammes per millilitre.

4.4 Discussion

There exists within asthma a sub-population of patients who fail to respond adequately to current therapies (25, 29). As a result this group have worse asthma control and consume a disproportionate share of healthcare budgets. Smokers with asthma comprise part of this difficult to control group. Previous research has demonstrated that the therapeutic response to inhaled corticosteroids is impaired in smokers with asthma (5-10). A recent post hoc analysis suggests that smokers with asthma also fail to gain the expected response to the combination of inhaled steroids and long acting beta agonists (compared to non smokers with asthma)(10). This randomised, controlled, exploratory clinical trial examined the impact of a novel alternative approach using the PPARγ agonist rosiglitazone, in a group of smokers with mild to moderate asthma.

Treatment with rosiglitazone produced a trend to improvement in pre-bronchodilator FEV_1 over low dose inhaled beclometasone at both 14 and 28 days. This improvement is much larger than the effect seen in previous trials in smokers with asthma examining inhaled corticosteroids (7, 8) and was associated with an improvement in PEF (albeit non-significant) and a significant improvement in the spirometric marker of small airway function FEF_{25-75}. The improvements in the pre-defined secondary endpoints suggest that the change observed in FEV_1 is real. The failure to produce a conclusive improvement relative to inhaled beclometasone is likely to be due to underpowering for the primary endpoint. A post hoc power calculation based on a standard deviation of 286ml and power of 80% with α set at 5% suggests that 40 patients per group would have been required for sufficient powering for this endpoint.

The improvement seen in FEF_{75} at 14 days and FEF_{25-75} at both 14 and 28 days is of interest as there are few therapies available for the treatment of small airway obstruction. Small airway obstruction, seen in many pulmonary conditions including asthma (269), the smoking related condition COPD (270) and several interstitial lung diseases (271), is associated with dynamic hyperinflation, reduced exercise tolerance and increased dyspnoea. Given the observed

improvement consideration should be given to studying PPARγ agonists in subjects with evidence of small airways obstruction.

A surprising finding was that the improvement in lung function produced by rosiglitazone was not associated with a reduction in asthma symptoms (as detected by the ACQ score) or sputum profile or supernatant at 28 days. What can explain this discrepancy? With regards the lack of sputum change the subjects had relatively mild asthma and did not display sputum eosinophilia or neutrophilia at baseline. This would explain the lack of change in the proportion of these inflammatory cells and possibly the lack of change in sputum cytokines. One explanation for the lack of change in ACQ score is that the lung function change is a random chance event given the small number of subjects studied. However the secondary lung function endpoints demonstrate a similar improvement so whilst a random chance event is possible other explanations are worth consideration. A possible alternative is that we are observing dissociation between lung function improvements and change in the asthma control questionnaire score. Previous examples include a study comparing two inhaled steroid preparations which examined improvements in lung function and symptoms (as measured by the asthma quality of life questionnaire) (272). The study found a clear difference in asthma symptom control between the two preparations despite equivalence between the treatments for lung function changes. When examined, the asthma symptom change was found to correlate poorly with lung function changes (272). This dissociation between asthma symptoms measured by questionnaire and lung function change has been observed in other studies (273, 274). An additional alternative explanation for the lack of change in ACQ score is a waning of the beneficial effect of rosiglitazone on lung function by 28 days and hence a lack of detectable effect on asthma symptoms. This could be supported by the reduction in the size of in PEF, FEF_{25-75} and FEF_{75} differences between the rosiglitazone and inhaled beclometasone arms from 14 to 28 days. This waning of effect, if true, could be due to tachyphylaxis. Previous research has demonstrated a down-regulation of PPARγ expression in asthmatics following treatment (117) and future research should follow PPARγ expression in endobronchial specimens during and after treatment with PPARγ agonists.

What are the mechanism(s) by which rosiglitazone could be producing the observed improvements in lung function? The cause of the poor response to corticosteroids in smokers with asthma is currently unknown. However one possible reason is that cigarette smoking induces an oxidative stress mediated change in the glucocorticoid receptor, resulting in a change in its behaviour and efficacy (32). Recent research has demonstrated that rosiglitazone is able to bind to the glucocorticoid receptor ligand binding domain with properties suggestive of a partial agonist effect (177). Therefore the presented results may be a demonstration of an alternative mode of glucocorticoid receptor activation which has resulted in the detected improvements in lung function. Alternatively, PPARγ has been shown to modulate a distinct but partially overlapping set of inflammatory genes compared to corticosteroids (163). Further studies examining the relative effects of rosiglitazone on corticosteroid and PPARγ-specific functional outputs are indicated in smokers with asthma and other conditions with relative corticosteroid insensitivity.

Only one dose of rosiglitazone was employed in this study. This was due to the exploratory nature of the trial and the lack of previous data on the efficacy of rosiglitazone in asthma. The dose selected is in common use for the treatment of non-insulin dependent diabetes and within the dose range used in models of asthma. Given the suggestion of a response, future trials should incorporate a number of different doses to examine the lung function dose response. Another aspect of PPARγ stimulation not examined in this trial is the potential synergistic interaction between PPARγ and the glucocorticoid receptor (38, 176). Previous research suggests that PPARγ may be able to modulate glucocorticoid receptor function and hence in circumstances of glucocorticoid insensitive inflammation may restore corticosteroid sensitivity. Therefore future trials should also examine combinations of PPARγ agonists and corticosteroids to determine if there is a useful synergistic effect with this combination.

Polymorphisms in the PPARγ receptor have recently been examined in a group of young subjects with asthma (178). Several single nucleotide polymorphisms were examined in this study and one common SNP combination, the ProC phenotype, was associated with increased asthma exacerbations and hospital admissions. Unfortunately PPARγ SNPs were not examined in this study and therefore the

role of SNPs in treatment response cannot be examined here. Clearly SNPs affecting the PPARγ expression level and behaviour may be of relevance to treatment response and future work should address the relevance of PPARγ receptor polymorphisms to response to treatment with PPARγ agonists.

4.5 Conclusion

This exploratory trial, the first to examine a PPARγ agonist in asthma, has demonstrated improvement in some lung function parameters in a group of smokers with mild to moderate asthma. The conclusions that can be drawn from the study are tempered by the exploratory nature of this work, reflected in the short duration of treatment and small number of subjects involved. However the results presented provide sufficient information for an adequately powered trial of this therapy in smokers with asthma and are encouraging given the documented poor response of smokers with asthma to standard doses of inhaled corticosteroids and the need for more effective therapies in this group. Further trials should be undertaken to examine PPARγ agonists in asthma and other obstructive airway conditions. PPARγ agonists may represent a new therapeutic class for inflammatory diseases.

5 Impact of smoking on cytokine profiles in asthma

5.1 Introduction

The cytokine family acts as a system of communication and control within and between the innate and adaptive immune system. Over 100 cytokines are recognised and many have important roles in the development and persistence of chronic inflammatory diseases. Multiple cell types from the innate and adaptive immune system express and respond to cytokines and there is considerable overlap in cytokine production. For example interleukin (IL)-13 can be produced by T lymphocytes, mast cells, eosinophils and basophils and IL-6 can be produced by macrophages, bronchial epithelial cells, T cells and B cells. Several cytokines have been linked to the recruitment and continued activation of inflammatory cells within the airway lumen and bronchial walls and are being targeted by pharmaceutical companies as potential therapeutic targets as a result (275).

Despite the crossover in expression that exists, cytokine profiles can be useful in characterising inflammation based on the expression of certain sets of cytokines by certain inflammatory cells. The classic example is the division of T lymphocyte CD4 helper cells into Th1 and Th2 subsets. Th1 cells develop from naive T helper cells in response to IL-12, interferon-γ (IFNγ) and transforming growth factor ß (TGFß) and produce IFNγ, TGFß and IL-2. Th2 cells differentiate in response to IL-4 and produce IL-4, 5, 6, 9, 10, 13 and 25.

Asthma, when introduced from an immunological perspective, is commonly portrayed as a prototypic Th2 disease. Whilst some evidence obtained from atopic subjects with asthma is available to support this view, it is an obvious oversimplification as it does not reflect the breadth of the inflammatory response in asthma, which is best described as heterogeneous (276, 277). The best current anti-inflammatory therapy for asthma is inhaled corticosteroids. Corticosteroids are effective in reducing eosinophilic inflammation, a range of inflammatory cytokines and provide a degree of asthma symptom control in the

majority of subjects. However not all subjects with asthma gain the expected benefits from this treatment and this probably reflects variations in the inflammatory response within asthma. Cigarette smoking is recognised to modulate the response to treatment with corticosteroids in asthma (261). This clinical observation suggests an altered immune response may be present in this sub group. Cytokines can alter corticosteroid responses, as demonstrated by previous *in-vitro* research using IL-2 & 4 to induce corticosteroid resistance in T lymphocytes (66-69). Previous research in smokers with asthma has identified increased sputum IL-8 (41) and reduced sputum IL-18 (42) (compared to matched non smokers with asthma). However no further information is available on differences in cytokine profiles in smokers with asthma compared to non-smoking asthmatics.

Recent developments allow the detection of multiple cytokines simultaneously in a small volume of sample. This unbiased approach permits the examination of a wide range of cytokines, providing increased levels of discrimination between different types of inflammatory diseases and more closely reflects the situation *in-vivo*. Therefore a cross sectional study was undertaken to obtain samples to examine the hypothesis that smokers with asthma have a reduced response to corticosteroids due to increased levels of IL-2 and 4 and that smokers with asthma display a generally altered cytokine profile in sputum and plasma compared to non-smokers with asthma.

5.2 Methods

5.2.1 Subjects

Subject characteristics, inclusion/exclusion criteria and recruitment methods are as described in the general methods chapter. All subjects provided informed consent and the study was approved by the West Glasgow Ethics Committee.

5.2.2 Study design

A full description of the study is provided in the general methods chapter. In brief, the study was a cross-sectional design with unblinded use of oral dexamethasone to determine corticosteroid sensitivity.

5.2.3 Measurements

Sputum induction and processing were as previously described in the general methods chapter. Briefly the whole sputum sample method was used and homogenisation was via mechanical processing with reduced levels of dithiothreitol. Sputum supernatants were collected post processing for sputum differential counts and stored in aliquots at -80°C until processing. Plasma was from heparinised blood samples. Subject demographics, baseline spirometry pre and post inhaled β_2 agonist and pre and post oral corticosteroid trial, asthma control questionnaire (ACQ) score and exhaled nitric oxide levels ($F_{E_{NO}}$ performed at flow rate 50ml/sec) were used for the analyses reported in this chapter. Baseline results for all subjects were examined. Compliance with oral dexamethasone was confirmed by suppression of plasma cortisol below 50nmol/l. If this criterion was met then the subject's data post corticosteroid data was analysed.

Initial cytokine analysis was performed using a 25-plex cytokine assay (Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, UK). Through the use of spectrally encoded antibody conjugated beads, this assay can simultaneously detect eotaxin, granulocyte/monocyte-colony stimulating factor (GM-CSF), interferon-α (IFN-α), interferon-γ (IFN-γ), interleukin (IL) 1-receptor-antagonist (IL-1RA), IL-1β, IL-2, IL-2 receptor (IL-2R), IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, Interferon-inducible Protein of 10 kDa (IP-10 aka CXCL10), Monocyte Chemotactic Protein 1 (MCP-1), Monokine Induced by IFN-γ (MIG aka CXC9), Monocyte Inflammatory Protein 1α (MIP-1α aka CCL3), Monocyte Inflammatory Protein 1β (MIP-1β aka CCL4), Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES aka CCL5), and Tumour Necrosis Factor-α (TNF-α). The assay allows for the detection of each individual cytokine in a single sample due to the unique fluorescent properties of

the individual bead with reference to a standard curve for each cytokine. The signal output and hence cytokine concentration was determined on a Luminex 100^{TM} analyser (Luminex Corporation, 12212 Technology Blvd, Austin, Texas, USA) by interpolation into a standard curve made up of standards of known concentration. The determined concentration of some cytokines, when below the lowest standard concentration but greater than zero, was accepted if the regression obtained from the standards was linear in that working range and was of sufficient gradient to allow for confident extrapolation. Otherwise the concentrations were accepted to be half of the lowest standard rather than zero in order that these values could be included. Sputum cytokines were performed in all groups and plasma cytokine measurements in smokers and non-smokers with asthma only. No reproducibility testing was performed for the Luminex assay.

A high sensitivity ELISA for IL-6 (Abcam plc, Cambridge, UK) was employed to examine and corroborate the Luminex findings for this cytokine. Briefly, samples were diluted based on the Luminex result to ensure they were within the working range of the assay (0.8 to 50 pg/ml). One hundred microliters of sample was added to an equal volume of diluent in the pre-coated wells of the EILSA plate followed by fifty microliters of biotinylated anti-IL-6. The samples were then gently shaken for three hours at room temperature. The plate was then washed and horse-radish peroxidase solution was added to each well followed by gentle shaking for thirty minutes. The plate was then washed followed by the addition of tetramethylbenzidine solution. The plate was covered for five minutes and then read on a plate reader with 450nm as the primary wavelength and 620nm as the reference immediately after the application of stop solution (sulphuric acid). No reproducibility testing was performed.

5.2.4 Statistical analysis

Parametric data was assessed using t testing and non parametric using Mann-Whitney testing. All comparisons are between smokers and non smokers. Ex-smokers were not included in the formal comparison analyses due to the small number of subjects but some basic significance tests were performed. Correlations were performed using Spearman rank correlation testing (result

derived by this method designated by ρ) and Pearson correlation (result derived by this method designated by r). Adjustment for multiple comparisons was not performed routinely as all data was treated as exploratory. Correction where performed was by the Bonferonni method. α was set at 0.05. Analysis was performed on SAS v 9.1 (TS1M3) for Windows (SAS Institute Inc., NC, USA) and MINITAB 15 (Minitab Inc. State College, PA, USA).

5.3 Results

75 volunteers were screened for suitability. 22 smokers with asthma, 21 non smokers with asthma and 10 ex-smokers with asthma were recruited to the study. 20 smokers, 21 non smokers and 10 ex-smokers were able to provide a suitable sputum sample for analysis. 18 smokers, 9 ex-smokers and 16 non smokers with asthma completed the corticosteroid trial and were able to provide a sputum sample. 19 smokers and 20 non smokers with asthma provided plasma samples at baseline and 18 smokers and 17 non smokers with asthma provided a plasma sample at completion of the corticosteroid trial.

5.3.1 Baseline demographics

The recruited subjects were well matched for relevant clinical characteristics (tables 5.1, 5.2 & 5.3). Smokers with asthma had significantly higher daily inhaled corticosteroid dose and asthma control questionnaire scores. The bronchodilator response of smokers with asthma was lower than that observed in non-smokers with asthma. No significant difference was evident for baseline sputum eosinophil or sputum neutrophil percentage when smokers and non-smokers with asthma were compared (table 5.3).

5.3.2 Lung function response to oral corticosteroid trial

Non-smokers with asthma made a significant improvement in lung function in response to the oral corticosteroid trial (figure 5.1 & table 5.4). This was in contrast to smokers and ex-smokers with asthma who both failed to make a significant improvement in lung function in response to oral corticosteroids.

There was no significant difference present when change in lung function was compared between the groups.

	Smokers (n=22)	Ex-Smokers (n=10)	Non-Smokers (n=21)
Age (yrs)	46.6 (6.7)	49.8 (9.0)	42.5 (10.0)
Sex (F:M)	12:10	5:5	11:10
BMI (kg/m^2)	26.6 (6.0)	31.2 (5.3)	28.9 (5.1)
Asthma Duration (yrs)	22.1 (15.9)	24.6 (15.9)	28.6 (15.0)
Pack yrs	27.6 (15.7)	28.5 (15.9)	
Ex-smokers (no of yrs)		7.7 (4.5)	
Inhaled steroid (mcg/day)	1046 * (611)	1280 (551)	679 (419)
ACQ Score (0 to 6)	2.2 * (0.9)	2.3 (0.7)	1.5 (0.8)
Oral daily dexamethasone dose	6.6 (0.9)	7.3 (0.9)	7.1 (0.8)
Equivalent daily prednisolone dose (mg)	44.1 (6.1)	48.3 (6.1)	47.1 (5.0)

Table 5.1 Baseline demographics.

Data presented as mean (SD). *; p≤0.05.

	Smokers (n=22)	Ex-Smokers (n=10)	Non-Smokers (n=21)
Pre BD FEV_1 (litres)	2.24 (0.58)	2.47 (0.79)	2.43 (0.69)
Pre BD FEV_1 (% predicted)	73.6 (18.5)	79.7 (24.1)	73.3 (15.3)
Pre BD PEF (l/min)	360.5 (77.8)	388.0 (124.8)	399.1 (98.6)
Pre BD PEF (% predicted)	81.7 (20.8)	85.4 (24.7)	85.8 (19.1)
Pre BD FVC (litres)	3.33 (0.8)	3.63 (0.9)	3.68 (0.9)
Pre BD FVC (% predicted)	89.6 (12.8)	96.4 (19.8)	92.9 (12.2)
Pre BD FEV_1/FVC	68.1 (12.1)	67.5 (8.3)	65.9 (9.9)
Pre BD FEF_{25-75} (% pred)	44.8 (19.7)	49.2 (24.1)	42.7 (16.1)
FEV_1 BD response	15.1 * (8.5)	19.5 (17.7)	23.3 (15.9)

Table 5.2 Pre steroid lung function.

Data presented as mean (SD). *; p≤0.05.

	Smokers (n=20)	Ex-Smokers (n=10)	Non-Smokers (n=21)
Total cell count (10^6) mean (SD)	6.1 (6.5)	8.3 (9.8)	7.1 (14.8)
Eosinophils %	0.4 (0.0, 1.0)	1.0 (0.1, 5.0)	0.3 (0.0, 2.0)
Eosinophils (10^4)	2.0 (0.0, 4.0)	5.0 (1.0, 23.0)	1.0 (0.0, 7.0)
Neutrophils %	34 (24, 56)	37 (22, 63)	24 (11, 41)
Neutrophils (10^4)	125.0 (77, 240)	151.0 (99, 304)	106.5 (39, 178)
Macrophages %	37 (25, 61)	31 (27, 60)	45 (32, 61)
Macrophages (10^4)	168.0 (106, 243)	132.0 (82, 199)	121.0 (78, 254)
Lymphocytes %	0.1 (0, 0.6)	0.0 (0, 0)	0.0 (0, 0.5)
Lymphocytes (10^4)	1.0 (0.0, 3.0)	0.0 (0.0, 0.0)	0.0 (0.0, 2.0)
Bronchial epithelial cells %	16 (9, 26)	23 (12, 31)	23 (12, 31)
Bronchial epithelial cells (10^4)	59.0 (25, 98)	57.0 (24, 60)	55.0 (40, 109)

Table 5.3 Baseline sputum profiles.

Data presented as median (IQR) except where indicated

	Lung function response (FEV_1 (ml)) (95% CI)
Non-smokers with asthma	173 * (10, 336)
Ex-smokers with asthma	257 (-154, 667)
Smokers with asthma	32 (-115, 178)

Table 5.4 Within group lung function response to oral steroid.

Data presented as mean change (95% CI). *; p<0.05
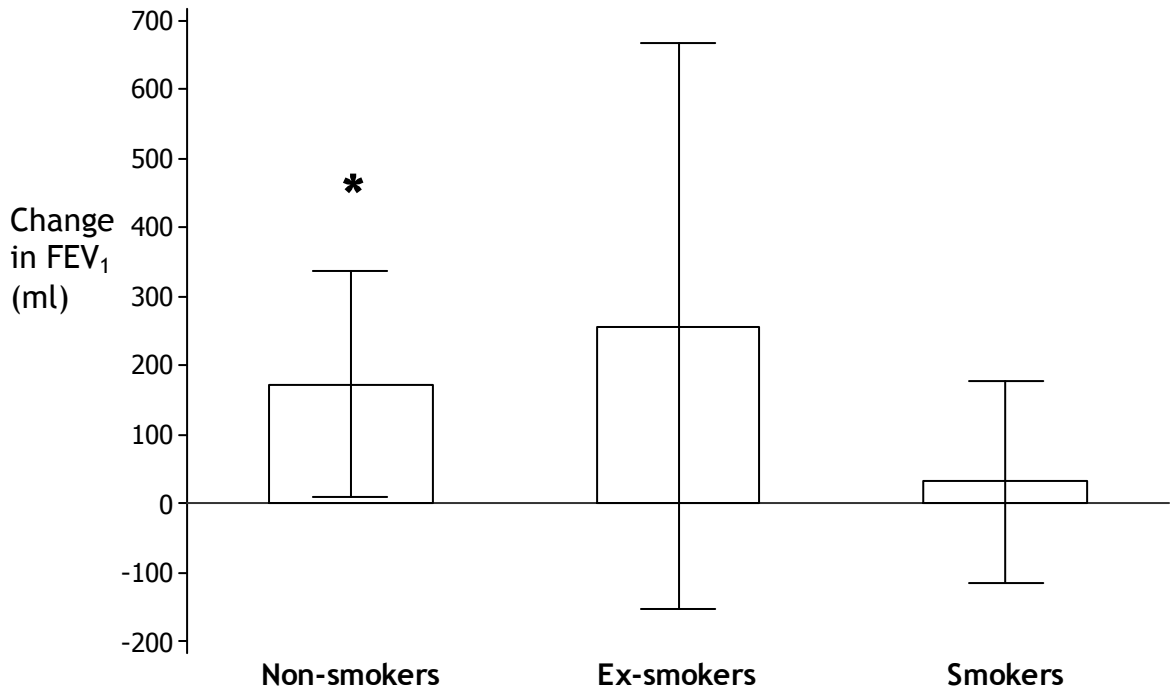
Figure 5.1 Within group change in FEV$_1$ in response to oral steroid trial.

Data presented as mean (95% CI). Interval bars represent 95% confidence intervals and mean. *; p<0.05.

5.3.3 Change in sputum cell profile in response to oral corticosteroid trial

All groups demonstrated significant within group eosinophil changes in response to oral corticosteroids. The eosinophil response of smokers with asthma to the corticosteroid trial was equivalent to that observed in non-smokers with asthma (smokers change -0.4% (95% CI -0.8, 0.0), non-smokers -0.2% (-2.0, 0.0), p=0.430). Smokers with asthma also demonstrated a trend to a reduction in sputum neutrophil percentage following oral corticosteroids (change -12.0% (-25.01, 1.99), p=0.081). However no difference was evident when compared to the neutrophil change observed in non-smokers. No significant changes were evident within or between the groups for the other sputum cell subtypes in response to the oral corticosteroid trial.

5.3.4 Change in asthma control questionnaire score

No improvement was detected in asthma symptoms as measured by asthma control questionnaire score (ACQ) in smokers with asthma in response to oral corticosteroids (change -0.1 (95% CI -0.7, 0.6), p=0.804). Non-smokers with asthma did demonstrate a reduction in ACQ score but this was less than the minimal clinically significant reduction of 0.5 (254) (change -0.4 (-0.7, -0.0), p=0.031). Ex-smokers demonstrated a large and significant reduction in ACQ score (change -1.0 (-1.8, -0.1), p=0.029). No significant difference was present when change in ACQ score in non-smokers and smokers with asthma was compared.

Change in ACQ score demonstrated a significant negative correlation with lung function improvement for non-smokers and ex-smokers with asthma. The correlation between ACQ score and lung function change in smokers with asthma demonstrated a trend to improvement:

- Non-smokers; r = -0.63, p=0.007

- Ex-smokers; r = -0.69, p=0.039

- Smokers; r = -0.42, p=0.062

5.3.5 Sputum supernatant cytokines

The majority of the sputum cytokines detectable by the Luminex assay were measurable in the majority of subjects (table 5.5). Expression levels were close to the limit of detectability for IL-4 in all groups, IFN-γ in non smokers and ex-smokers with asthma & IL-15 in non-smokers with asthma.

Smokers with asthma tended to a higher median concentration for all cytokines and had significantly higher levels (relative to non-smokers with asthma) for:

- IFN-γ (smokers with asthma 2.6 pg/ml (IQR 0.7, 7.7), non smokers 0.3 pg/ml (IQR 0.3, 0.3), p=0.025) (figure 5.2),

- IL-2 (4.4 pg/ml (3.7, 6.5), 3.6 pg/ml (3.3, 4.5), p=0.041) (figure 5.3),

- IL-4 (0.1 pg/ml (0.1, 4.9), 0.1 pg/ml (0.1, 0.1), p=0.038) (figure 5.4),

- IL-6 (34.4 pg/ml (14.1, 72.4), 8.1 pg/ml (4.4, 11.1), p<0.001) (figure 5.5),

- IL-7 (28.5 pg/ml (14.0, 65.6), 16.3 pg/ml (6.6, 18.8), p=0.044) (figure 5.6),

Sputum IL-12 and IL-17 levels demonstrated a trend to a difference between smokers and non smokers with asthma (IL-12; smokers 30.6 pg/ml (12.6, 49.6), non smokers 15.5 pg/ml (8.7, 22.2), p=0.050, IL-17; smokers 44.9 pg/ml (4.0, 146.1), non smokers 4.0 pg/ml (4.0, 16.3), p=0.080). Performing corrections for multiple comparisons resulted in all sputum cytokine differences losing statistical significance save sputum IL-6 (p=0.023).

Adjusting for inhaled corticosteroid dose did not remove any of the differences and strengthened several. For example sputum IL-12 and IL17 were significantly higher in smokers with asthma as a result of this change (IL-12; adjusted mean difference 37.2 pg/ml (95% CI 7.4, 67.1), p=0.016, IL-17; 102.4 pg/ml (14.9, 190.0), p=0.023).
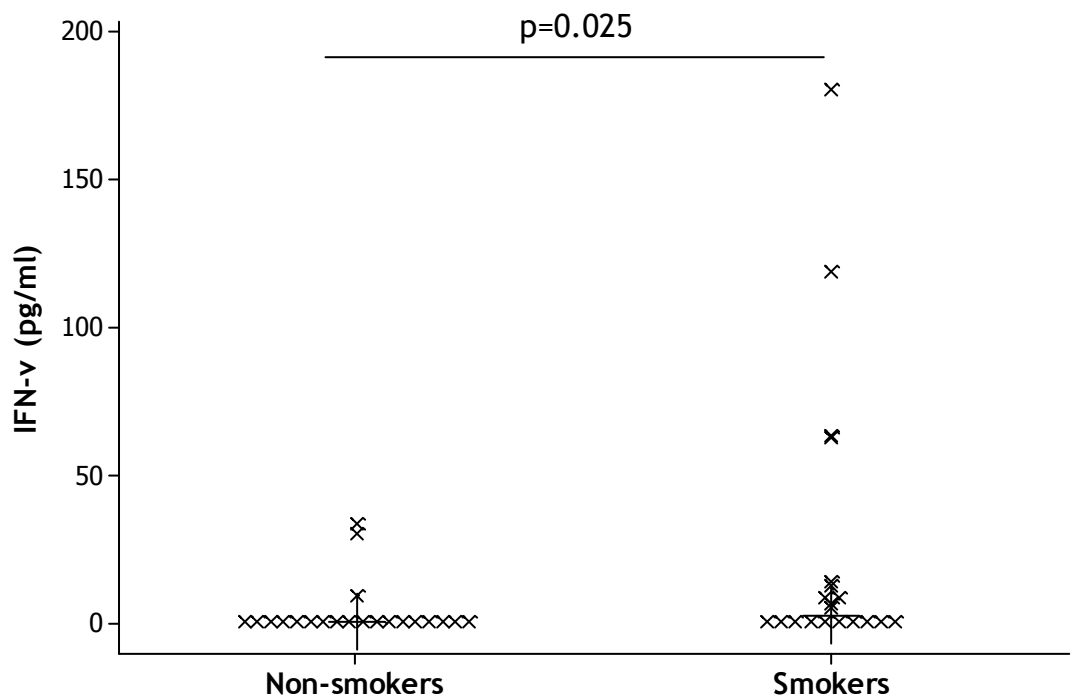
Figure 5.2 Baseline sputum supernatant IFNγ.

Data presented as individual points with median.

	Smokers	Ex-Smokers	Non-Smokers
Eotaxin	2.6 (0.7, 7.7)	4.6 (0.7, 5.5)	1.1 (0.3, 2.1)
GM-CSF	21.5 (8.4, 76.1)	20.1 (6.6, 96.9)	17.0 (6.6, 26.1)
IFN-α	24.5 (20.8, 55.5)	24.5 (20.8, 85.5)	20.8 (16.8, 27.9)
IFN-γ	2.6 * (0.3, 13.2)	0.3 (0.3, 25.0)	0.3 (0.3, 0.3)
IL-1RA	12480 (3475, 18561)	5205 (3808, 7943)	3448 (2173, 8428)
IL-1β	19.4 (14.1, 34.4)	16.7 (15.4, 42.1)	16.7 (12.7, 20.7)
IL-2	4.4 * (3.7, 6.5)	3.8 (3.3, 5.1)	3.6 (3.3, 4.5)
IL-2R	105.8 (11.9, 287.8)	163.7 (113.0, 568.1)	40.2 (11.9, 132.3)
IL-4	0.1 * (0.1, 4.9)	0.1 (0.1, 2.0)	0.1 (0.1, 0.1)
IL-5	4.7 (2.8, 10.6)	3.5 (3.1, 6.6)	3.1 (2.4, 4.2)
IL-6	34.4 * (14.1, 72.4)	34.9 (13.6, 148.9)	8.1 (4.4, 11.1)
IL-7	28.5 * (14.0, 65.6)	36.2 (14.5, 59.0)	16.3 (6.6, 18.8)
IL-8	1096 (398, 3059)	1715 (476, 5862)	650 (332, 1030)
IL-10	3.2 (1.8, 9.4)	3.2 (1.8, 6.4)	1.8 (1.5, 2.4)
IL-12	30.6 † (12.6, 49.6)	23.6 (12.6, 56.8)	15.5 (8.7, 22.2)
IL-13	29.4 (20.5, 47.6)	29.9 (22.5, 57.7)	24.4 (20.5, 28.1)
IL-15	13.5 (0.6, 45.8)	14.5 (0.6, 79.8)	0.6 (0.6, 7.0)
IL-17	44.9 (4.0, 146.1)	36.3 (4.0, 164.0)	4.0 (4.0, 16.3)
IP-10	52.6 (31.6, 104.5)	192.6 (54.0, 327.1)	59.8 (26.3, 90.2)
MCP-1	298.4 (162.5, 396.2)	317.2 (189.6, 452.6)	192.8 (140.6, 214.8)
MIG	127.9 (50.8, 239.3)	191.1 (94.4, 229.0)	124.8 (47.8, 146.5)
MIP-1α	26.9 (16.5, 62.9)	45.4 (29.0, 71.2)	20.8 (17.4, 29.0)
MIP-1β	30.1 (19.3, 86.7)	128.1 (43.0, 352.1)	27.8 (17.6, 38.8)
RANTES	42.8 (32.0, 64.1)	58.4 (38.1, 107.5)	37.3 (23.0, 44.9)
TNF-α	3.7 (1.7, 7.6)	4.0 (2.5, 6.5)	2.2 (1.9, 2.8)

Table 5.4 Sputum cytokine results-Baseline comparisons.

Data presented as median (IQR). All pg/ml. *; $p \leq 0.05$, †; $p = 0.05$
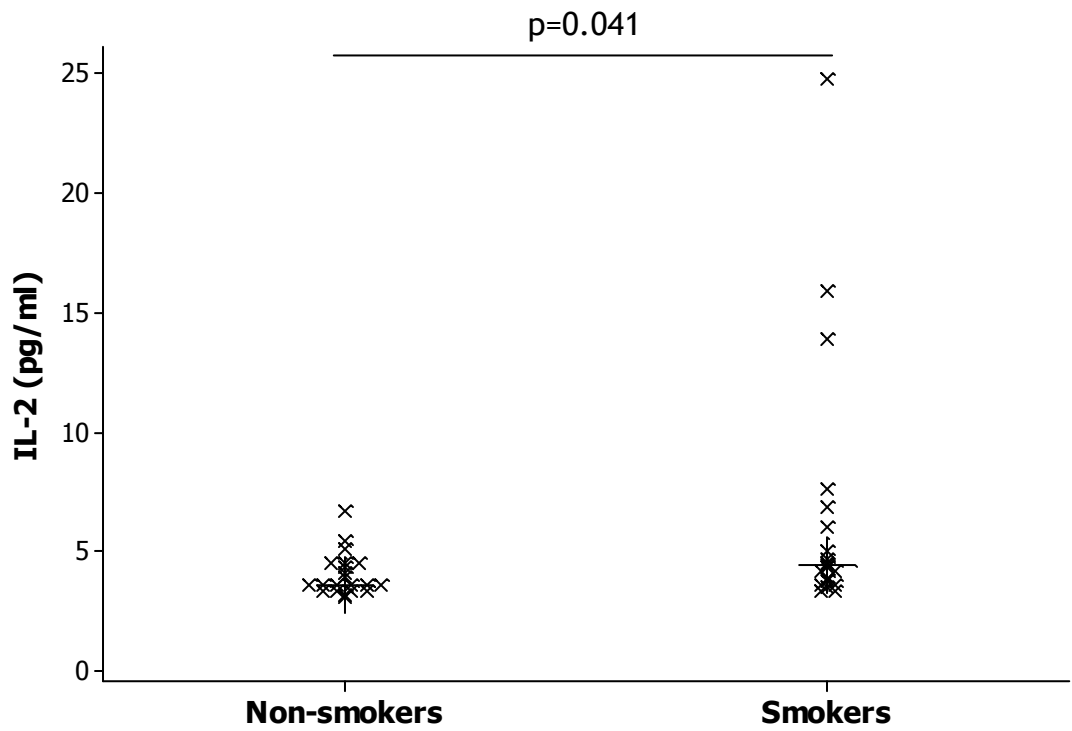
Figure 5.3 Baseline sputum supernatant IL-2.

Data presented as individual points with median.
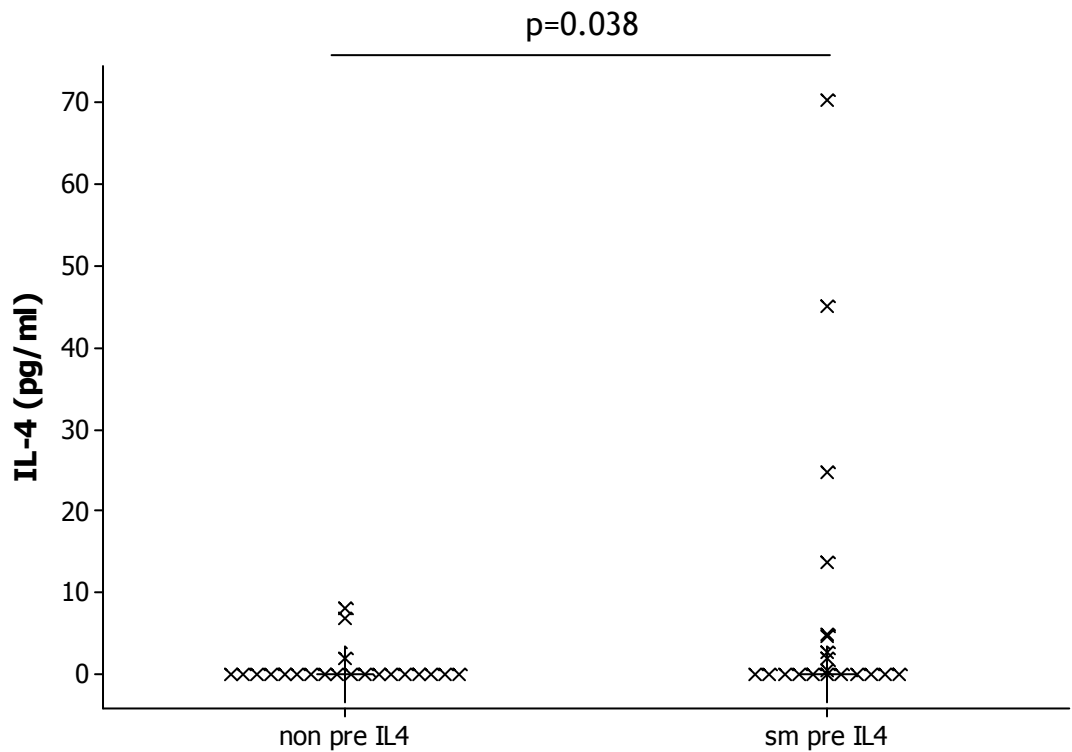
Figure 5.4 Baseline sputum supernatant IL-4.

Data presented as individual points with median.
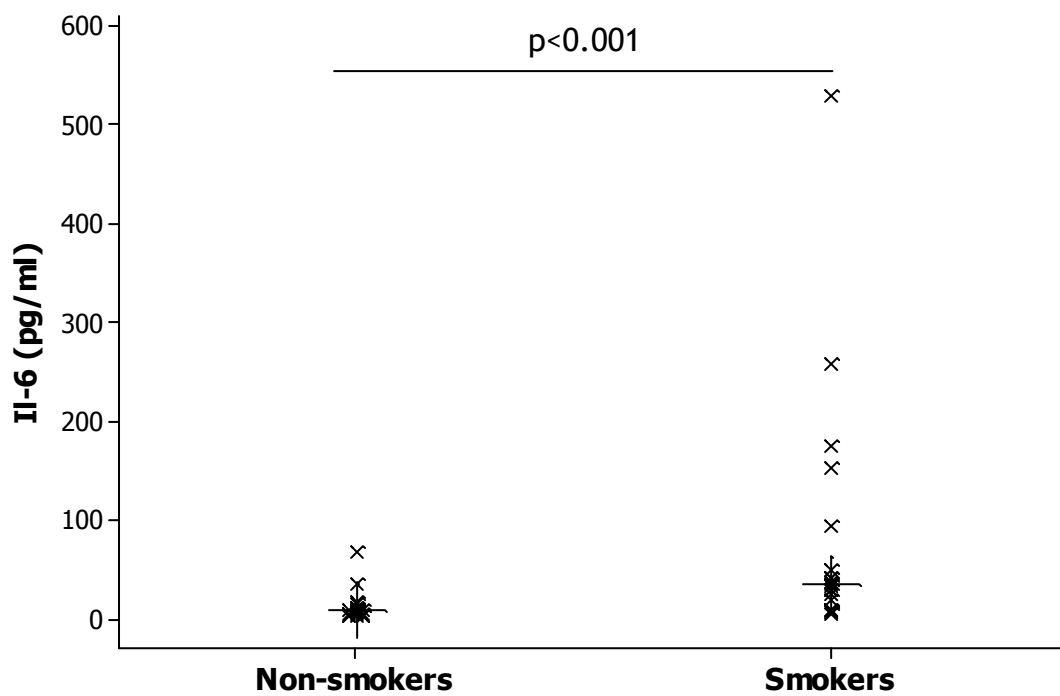
Figure 5.5 Baseline sputum supernatant IL-6.

Data presented as individual points with median.
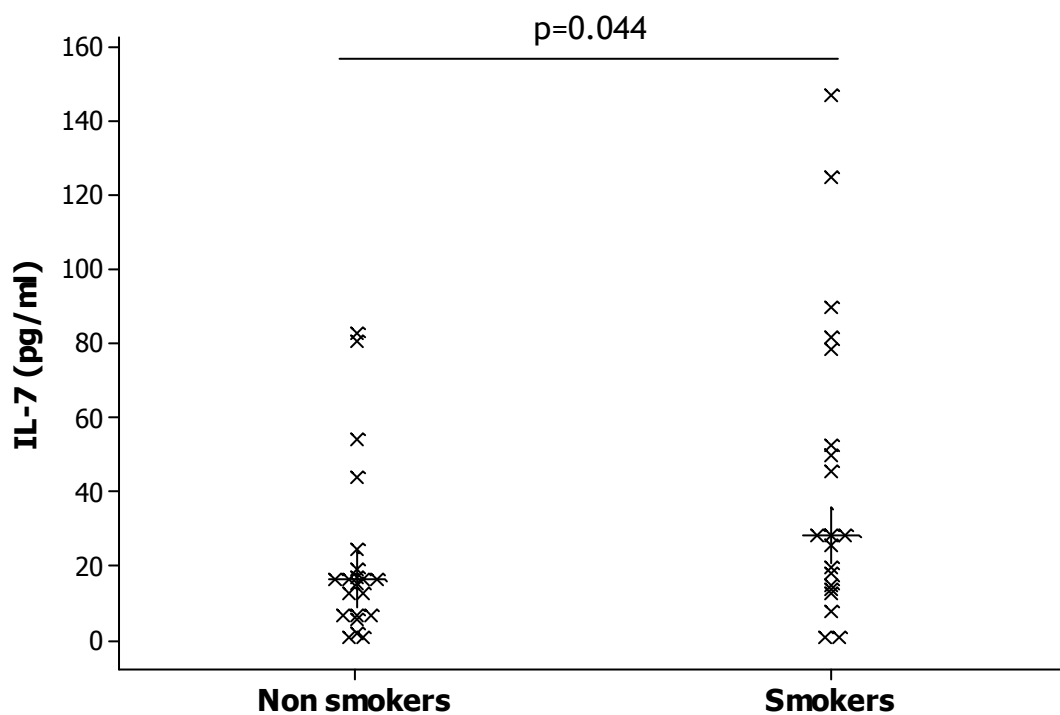
Figure 5.6 Baseline sputum supernatant IL-7.

Data presented as individual points with median.

5.3.5.1 Baseline sputum cytokine correlations

Smokers with asthma demonstrated a number of significant correlations between sputum cytokine concentrations and clinical endpoints:

- Sputum IL-6 and IL-8 demonstrated negative correlations with baseline pre-bronchodilator FEV_1:

    - IL-6; ρ= -0.47 (95% CI -0.75, -0.03), p=0.032 (Figure 5.7)

    - IL-8; ρ= -0.46 (-0.75, -0.03), p=0.034

- Sputum IL-6 and 8 also demonstrated positive correlations with subject age:

    - IL-6; ρ= 0.45 (0.01, 0.75), p=0.039

    - IL-8; ρ= 0.59 (0.21, 0.82), p=0.004

- Sputum IL-6 correlated negatively with exhaled nitric oxide (FE_{NO}) levels (ρ= -0.54 (-0.81, -0.08), p=0.020)

- Sputum IL-8 levels correlated positively with baseline ACQ score (ρ= 0.45 (0.01, 0.75), p=0.040)

- Sputum IL-8 correlated positively with pack years (ρ= 0.62 (0.25, 0.84), p=0.002)

- Sputum IL-1RA demonstrated a positive correlation with asthma duration (ρ= 0.44 (-0.01, 0.74), p=0.048)

- Sputum MIP1α and MIP1ß levels both correlated positively with subject age (MIP1α; ρ= 0.47 (0.03, 0.75) p=0.032, MIP1ß; ρ= 0.50 (0.07, 0.77), p=0.020)

No correlation was evident between sputum IL-6 and pack years in smokers with asthma (ρ=0.35 (-0.11, 0.69), p=0.124).
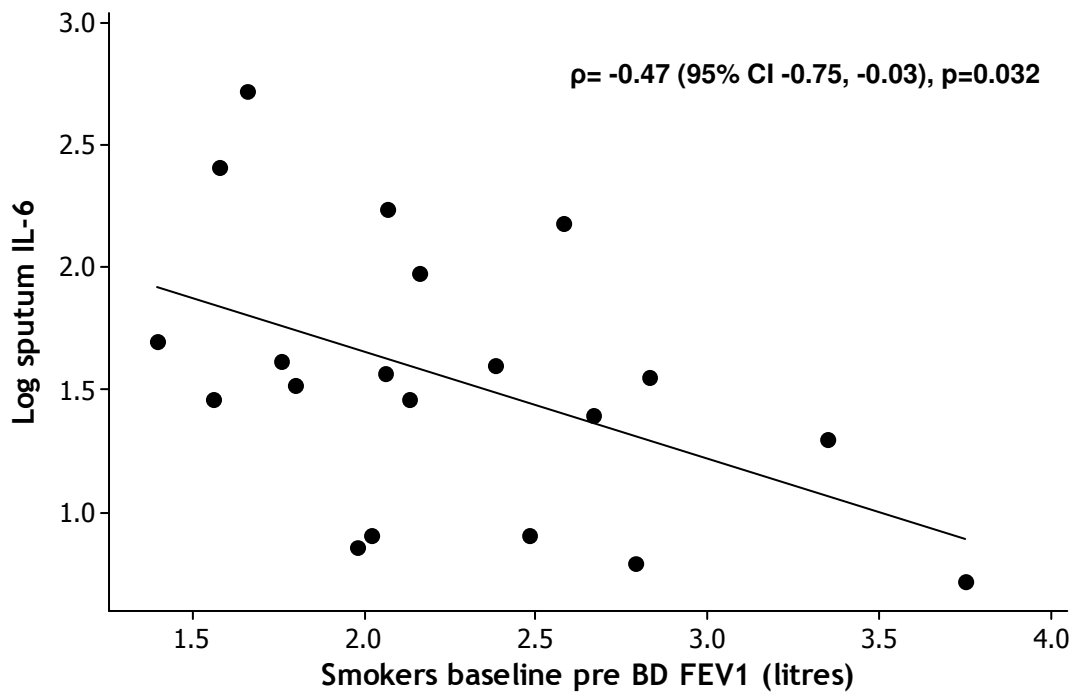
Figure 5.7 Scatter plot of smokers with asthma pre-steroid sputum Log IL-6 against pre-bronchodilator pre-corticosteroid trial FEV$_1$.

Sputum cytokines from non smokers with asthma also demonstrated correlations with some clinical endpoints:

- Sputum IFNγ correlated positively with baseline pre-bronchodilator FEV$_1$ (ρ= 0.44 (0.01, 0.73), p=0.041), and negatively with percentage change in FEV$_1$ in response to corticosteroids (ρ= -0.53 (-0.80, -0.09), p=0.018)

Multiple negative correlations were evident between sputum cytokines from non smokers with asthma and asthma duration:

- IL-1β; ρ= -0.48 (-0.75, -0.05), p=0.025

- IL-2; ρ= -0.52 (-0.78, -0.12), p=0.012

- IL-5; ρ= -0.66 (-0.85, -0.33), p<0.001

- IL-10; ρ= -0.62 (-0.83, -0.26), p=0.002

- IL-13; ρ= -0.42 (-0.72, 0.01), p=0.049

- GM-CSF; ρ= -0.47 (-0.75, -0.04), p=0.029

- IFNα; ρ= -0.59 (-0.81, -0.21), p=0.003

- MIP1α; ρ= -0.56 (-0.80, -0.16), p=0.007

- MIP1β; ρ= -0.46 (-0.75, -0.04), p=0.029

Sputum cytokines from ex-smokers with asthma also demonstrated a number of correlations. Negative correlations were present between sputum IL-8 and MCP-1 and FE_{NO}:

- IL-8 and FE_{NO}; ρ= -0.71 (-0.93, -0.09), p=0.022

- MCP-1 and FE_{NO}; ρ=-0.69 (-0.93, -0.06), p=0.027

Positive correlations were present between IL-6, MIP1α, MIP1β and baseline ACQ score in ex-smokers with asthma:

- IL-6 and ACQ; ρ= 0.68 (0.09, 0.92), p=0.021

- MIP1α and ACQ; ρ= 0.63 (-0.01, 0.90), p=0.042

- MIP1β and ACQ; ρ= 0.67 (0.08, 0.92) p=0.023

IL-6 and asthma duration also positively correlated (ρ= 0.67 (0.07, 0.91), p=0.024) and a strong correlation was present between sputum IL-6 and pack years (ρ= 0.68 (0.09, 0.92), p=0.022) in ex-smokers with asthma.

5.3.6 Baseline plasma cytokines

Several differences in median plasma cytokine concentrations were evident when smokers and non smokers with asthma were compared (table 5.6). Smokers demonstrated significantly reduced median plasma levels of IL-1RA, 10 & 13 and GM-CSF:

- IL-1RA; smokers 209 pg/ml (IQR 160, 252) non-smokers 247 pg/ml (IQR 224, 279), p=0.024 (Figure 5.8)

- IL-10; smokers 1.5 pg/ml (1.4, 1.7), non smokers 1.7 pg/ml (1.5, 2.6), p=0.027 (Figure 5.9)

- IL-13; smokers 19.5 pg/ml (17.4, 21.5), non smokers 21.5 pg/ml (20.0, 23.4), p=0.004 (Figure 5.10)

- GM-CSF; smokers 6.6 pg/ml (5.1, 13.0), non smokers 10.3 pg/ml (10.3, 26.1), p= 0.028 (Figure 5.11)

A borderline significant difference in plasma IL-12 was also present (smokers 69.1 pg/ml (46.0, 76.1), non-smokers 70.9 pg/ml (64.3, 85.7), p=0.053). Correcting for multiple comparisons resulted in all plasma cytokine differences losing statistical significance.
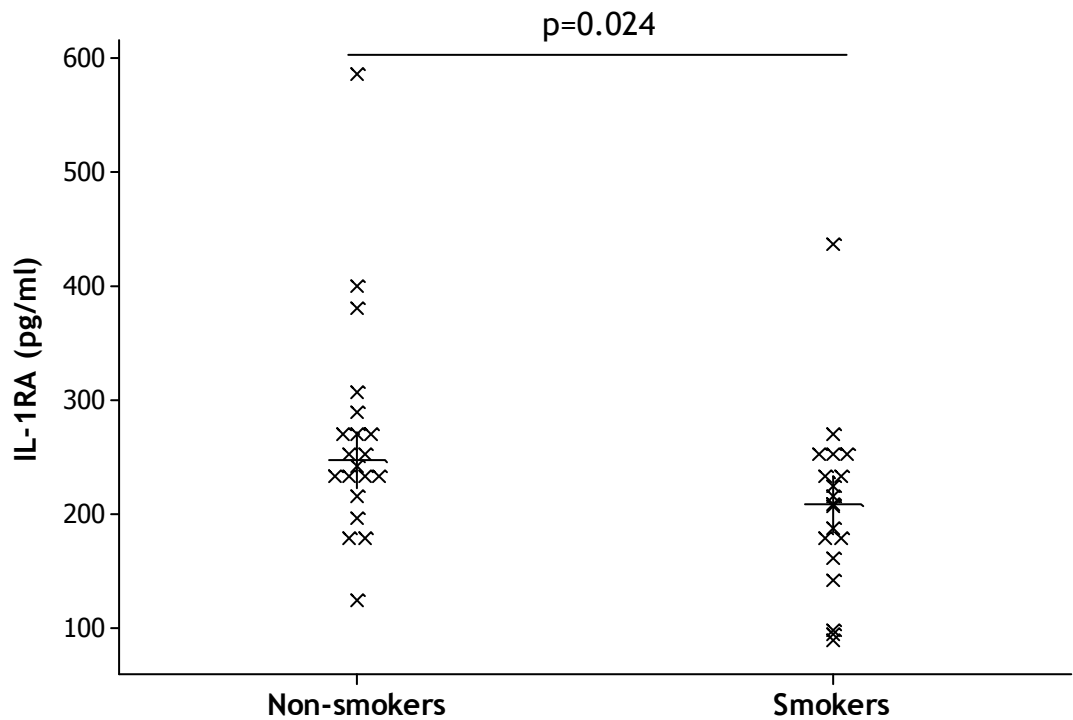
Figure 5.8 Baseline plasma IL-1RA levels in non-smokers with asthma and smokers with asthma.

Data presented as individual points with median.

	Smokers	Non Smokers
Eotaxin	88.8 (50.7, 129.5)	75.4 (42.7, 86.0)
GM-CSF	6.6 * (5.1, 13.0)	10.3 (10.3, 26.1)
IFN-α	31.2 (24.5, 34.3)	32.7 (28.7, 38.6)
IFN-γ	2.0 (0.3, 4.2)	2.6 (0.6, 4.2)
IL-1RA	209 * (160, 252)	247 (224, 279)
IL-1β	13.4 (10.7, 20.1)	15.4 (12.7, 18.7)
IL-2	4.4 (3.7, 5.6)	5.0 (4.6, 6.6)
IL-2R	294 (165, 354)	251 (214, 306)
IL-4	9.7 (6.3, 11.3)	10.5 (8.7, 12.7)
IL-5	2.5 (2.2, 2.7)	2.5 (2.5, 2.9)
IL-6	1.5 (1.2, 2.4)	1.7 (1.3, 3.2)
IL-7	16.3 (7.7, 18.0)	19.7 (14.9, 25.0)
IL-8	5.1 (3.6, 6.8)	6.1 (3.6, 8.0)
IL-10	1.5 * (1.4, 1.7)	1.7 (1.5, 2.6)
IL-12	69.1 † (46.0, 76.1)	70.9 (64.3, 85.7)
IL-13	19.5 * (17.4, 21.5)	21.5 (20.0, 23.4)
IL-15	4.1 (0.6, 8.0)	7.5 (1.4, 9.9)
IL-17	22.6 (8.6, 47.7)	33.4 (16.3, 56.2)
IP-10	10.0 (8.1, 17.0)	14.5 (10.8, 17.9)
MCP-1	175 (116, 259)	165 (148, 234)
MIG	12.0 (12.0, 12.0)	12.0 (12.0, 13.7)
MIP-1α	24.1 (19.1, 25.7)	24.1 (24.1, 27.3)
MIP-1β	27.8 (24.4, 31.3)	31.3 (27.2, 35.3)
RANTES	4639 (3654, 6269)	6387 (4300, 9426)
TNF-α	2.7 (2.1, 2.9)	2.9 (2.5, 3.2)

Table 5.6 Baseline plasma cytokines.

All pg/ml. Expressed as median (IQR). *; $p \leq 0.05$, †; p=0,05
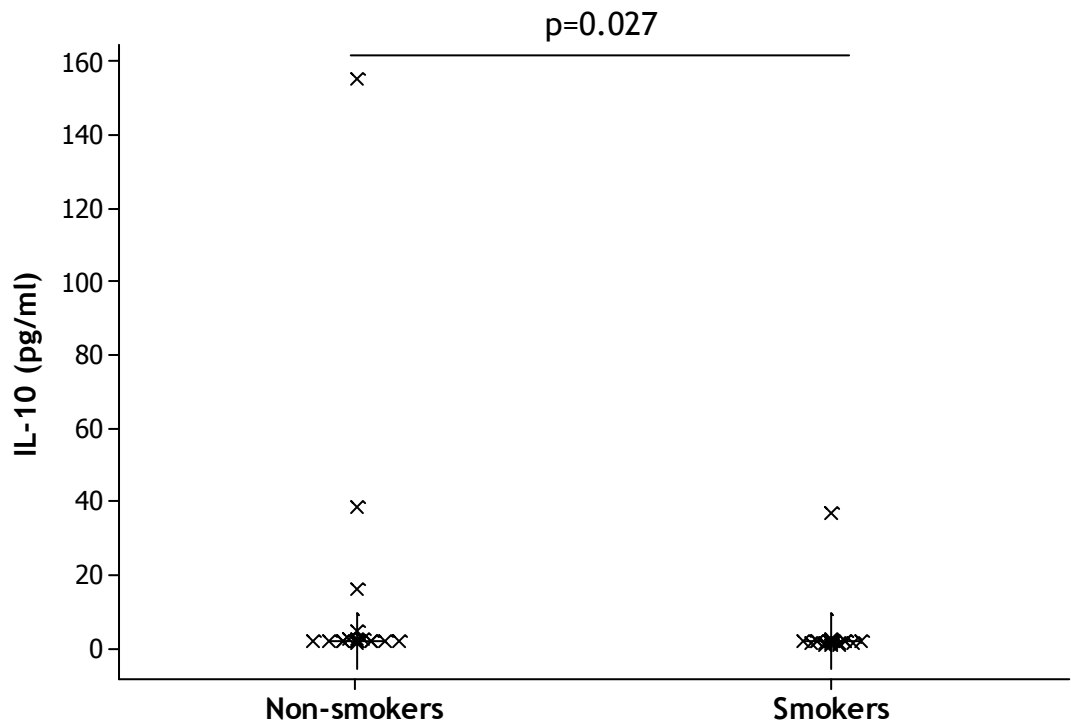
Figure 5.9 Baseline plasma IL-10 levels in non-smokers with asthma and smokers with asthma.

Data presented as individual points with median.
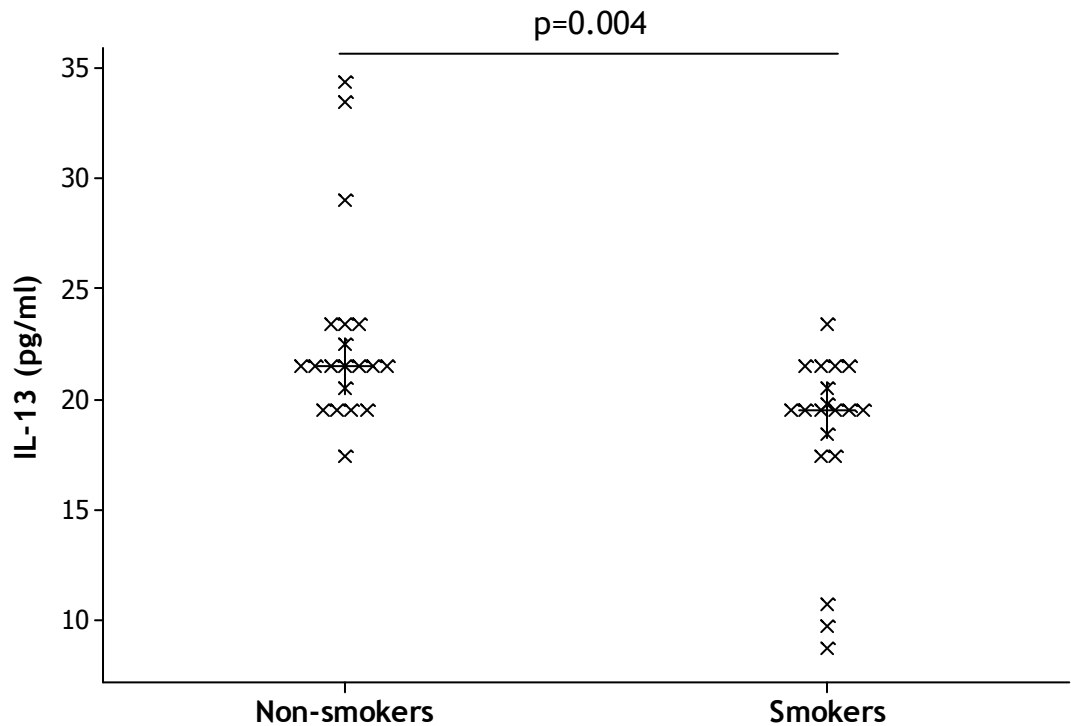
Figure 5.10 Baseline plasma IL-13 levels in non-smokers with asthma and smokers with asthma.

Data presented as individual points with median.
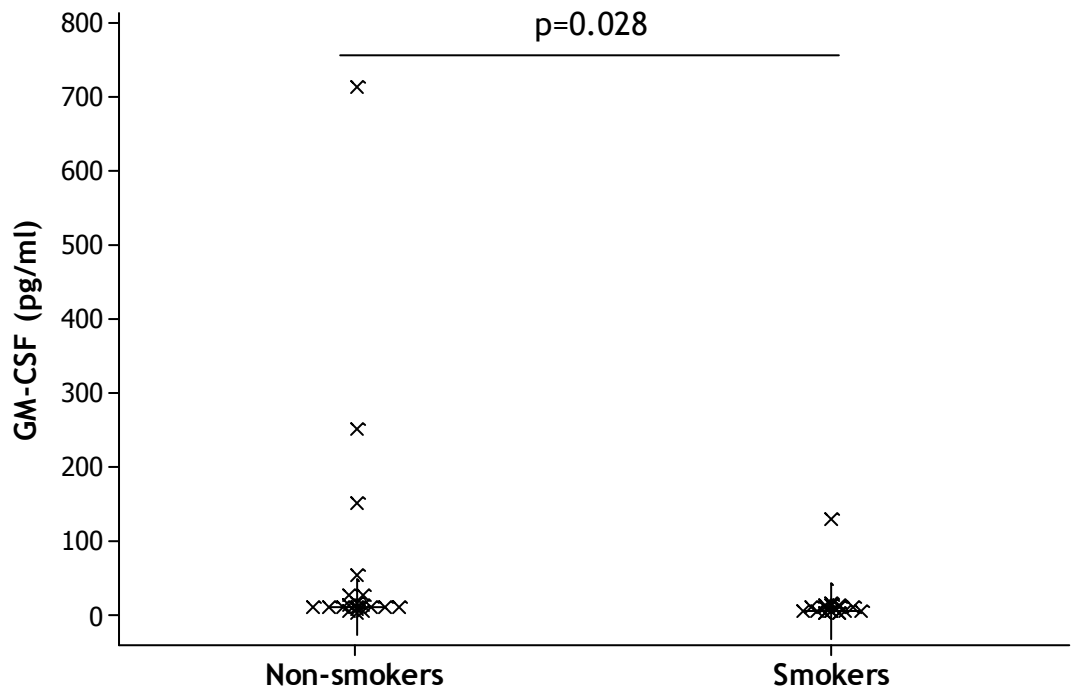
Figure 5.11 Baseline plasma GM-CSF levels in non-smokers with asthma and smokers with asthma.

Data presented as individual points with median.

5.3.6.1 Baseline plasma cytokine correlations

A number of correlations were present between baseline plasma cytokines and clinical characteristics.

IL2 and IL2R correlated with pre corticosteroid pre BD FEV_1 in smokers with asthma:

- IL-2; ρ= -0.46 (95% CI -0.76, -0.01), p=0.040

- IL-2R; ρ= -0.62 (-0.84, -0.24), p=0.003

A trend to statistical significance was evident for the correlation between plasma IL-6 and IL-12 and pre corticosteroid pre BD FEV_1 in the smokers with asthma:

- IL-6; ρ= -0.42 (-0.73, 0.05), p=0.068

- IL-12; ρ= -0.39 (-0.72, 0.07), p=0.087

Multiple correlations were also evident between baseline plasma cytokines from smokers with asthma and FEV_1 response to oral corticosteroid:

- IFN-α; ρ= 0.45 (-0.01, 0.75), p=0.049

- IL-1RA; ρ= 0.68 (0.33, 0.87), p<0.001

- IL-1β; ρ= 0.62 (0.23, 0.84), p=0.003

- IL-2; ρ= 0.58 (0.17, 0.82), p=0.007

- IL-5; ρ= 0.60 (0.20, 0.83), p=0.004

- MIP-1α; ρ= 0.48 (0.03, 0.77), p=0.033

- IL-12; ρ= 0.55 (0.13, 0.80), p=0.011

- IL-15; ρ= 0.63 (0.25, 0.84), p=0.002

Further correlations were observed between $F_{E_{NO}}$ and smokers with asthma plasma cytokines:

- IL-17; ρ= 0.60 (0.17, 0.84), p=0.008

- RANTES; ρ= -0.59 (-0.83, -0.15), p=0.009

No correlation was evident between plasma IL-6 and pack years in the smokers with asthma (ρ= -0.31 (-0.67, 0.17), p=0.194).

Non smokers also demonstrated correlations for plasma cytokines but these were with different clinical parameters. Correlations existed between ACQ score at baseline and non smokers with asthma plasma cytokines for:

- IL-8; ρ= 0.44 (0.00, 0.74), p=0.043

- MCP-1; ρ= 0.57 (0.16, 0.81), p=0.007

A borderline significant correlation was also present between IFNγ and baseline ACQ score (ρ= 0.43 (-0.02, 0.73), p=0.055). Non smokers with asthma

demonstrated a significant correlation between plasma IFNγ and subject age (ρ= -0.48 (-0.76, -0.05), p=0.027) and several correlations between plasma cytokines and asthma duration:

- IL-2; ρ= -0.52 (-0.78, -0.10), p=0.015

- MCP-1; ρ= -0.45 (-0.74, -0.01), p=0.041

- MIP-1β; ρ= -0.44 (-0.74, 0.00), p=0.047

A borderline correlation was also evident between IL-1β and asthma duration (ρ= -0.42 (-0.73, 0.02), p=0.056).

5.3.7 Correlation between sputum cytokines

When sputum cytokine relationships were examined, using the data from all groups combined, a number of strongly significant relationships were evident. Sputum IL-2 & 4 demonstrated a strong correlation (ρ=0.73, p<0.001) and IFNγ correlated strongly with IL-2 (ρ=0.74, p<0.001), IL-4 (ρ=0.95, p<0.001) and IL-12 (ρ=0.79, p<0.001). Sputum IL-6 correlated weakly with IL-17 (ρ= 0.29, p=0.039) and strongly with IL-8 (ρ= 0.70, p<0.001) and MCP-1 (ρ= 0.74, p<0.001).

5.3.8 Correlation between plasma and sputum cytokines

When the correlations between plasma and sputum cytokines from all subjects were examined only two statistically significant correlations were present. Plasma and sputum eotaxin demonstrated a positive correlation (ρ= 0.66 (95% CI 0.43, 0.81), p<0.001). Plasma and sputum RANTES demonstrated a negative correlation (ρ= -0.33 (-0.59, -0.01), p=0.041). A number of cytokines demonstrated borderline significant associations:

- IL-1RA; ρ= -0.28 (-0.55, 0.05), p=0.090

- IL-2; ρ= 0.29 (-0.04, 0.56), p=0.079

- IL-7; ρ= -0.30 (-0.57, 0.02), p=0.065

- MIG; ρ= 0.31 (-0.02, 0.57), p=0.061

- MIP1α; ρ= 0.29 (-0.04, 0.56), p=0.080

Non smokers with asthma demonstrated a significant correlation between plasma and sputum eotaxin (ρ= 0.54 (0.12, 0.79), p=0.011) and borderline significant correlations between plasma and sputum:

- GM-CSF; ρ= 0.38 (-0.08, 0.70), p=0.094

- IL-2; ρ= 0.39 (-0.06, 0.71), p=0.078

- IL-15; ρ= 0.40 (-0.05, 0.72), p=0.072

- MIG; ρ= 0.43 (-0.02, 0.73), p=0.055

- MIP1α; ρ= 0.40 (-0.05, 0.72), p=0.074

- MIP1β; ρ= 0.38 (-0.08, 0.70), p=0.092

Smokers with asthma also demonstrated a significant correlation between plasma and sputum eotaxin (ρ= 0.63 (0.22, 0.85), p=0.004) and further significant correlations for IL-1RA (ρ= -0.56 (-0.82, -0.11), p=0.015) and IL-2 (ρ= 0.51 (0.04, 0.80), p=0.029). No borderline significant correlations between plasma and sputum cytokines were evident for smokers with asthma.

5.3.9 Cytokine response to oral corticosteroid trial

5.3.9.1 Sputum cytokine responses

When examined post corticosteroid trial sputum median cytokine levels demonstrated significant differences between smokers and non smokers with asthma for IL-1RA and eotaxin and a failure of sputum IL-6 levels to normalise (table 5.7 and figure 5.12):

- IL1RA; smokers 16140 pg/ml (IQR 4208, 23359), non-smokers 4838 pg/ml (IQR 2626, 7892), p=0.033

- Eotaxin; smokers 4.2 pg/ml (1.5, 9.9), non-smokers 0.8 (0.2, 1.7), p=0.012

- IL-6; smokers 24.3 pg/ml (17.5, 74.2), non-smokers 7.3 (2.2, 21.1), p=0.027

A borderline difference was still present for sputum IL-12 (smokers 41.5 (20.3, 114.0), non smokers 18.4 (7.7, 52.7), p=0.080).
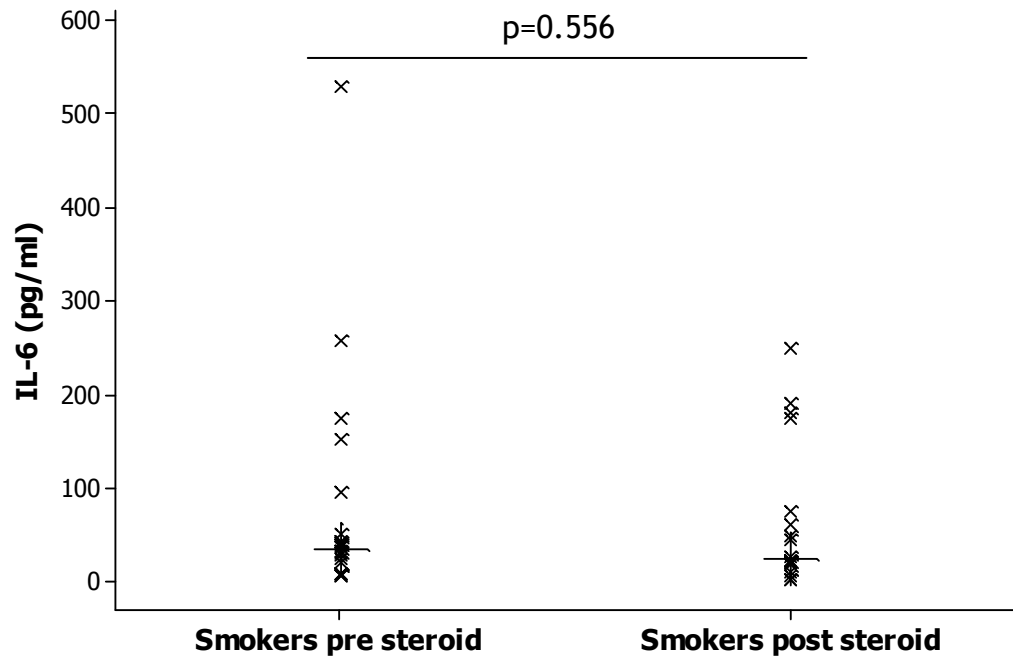
Figure 5.12 Comparison of pre steroid and post steroid sputum IL-6 in smokers with asthma Data presented as individual points with median.

Few statistically significant within group changes were evident in response to oral corticosteroids. Smokers with asthma demonstrated an increase in sputum IFNα (53.5 pg/ml (95% CI 12.1, 94.8), p=0.014) and IL-17 (119.6 pg/ml (30.7, 208.5), p=0.011). A smaller but non-significant increase was also evident for these cytokines in the non-smokers in response to the corticosteroid trial (IFNα; 24.5 pg/ml (-4.2, 53.3), p=0.089, IL-17; 67.4 pg/ml (-8.5, 143.3), p=0.078). An increase in MIP1β was detected in the non-smokers with asthma (31.6 pg/ml (4.6, 58.5), p=0.025). No significant differences were evident for change in sputum cytokines in smokers with asthma compared to non smokers with asthma.

	Smokers	Ex Smokers	Non Smokers
Eotaxin	4.2 * (1.5, 9.9)	1.9 (1.1, 6.1)	0.8 (0.2, 1.7)
GM-CSF	56.4 (10.3, 144.9)	38.6 (23.1, 180.2)	31.7 (8.4, 102.8)
IFN-α	58.5 (20.8, 137.2)	48.1 (27.9, 122.9)	31.2 (18.8, 74.2)
IFN-γ	6.2 (0.3, 47.4)	0.3 (0.3, 68.5)	0.3 (0.3, 26.9)
IL-1RA	16140 * (4208, 23359)	12168 (7528, 14239)	4838 (2626, 7892)
IL-1β	28.9 (14.7, 82.4)	62.4 (20.7, 83.0)	18.1 (12.4, 40.9)
IL-2	5.2 (3.6, 10.6)	4.5 (3.6, 11.8)	4.2 (3.3, 5.4)
IL-2R	132.1 (5.0, 834.3)	113.0 (40.2, 976.8)	60.3 (5.0, 529.9)
IL-4	3.5 (0.1, 22.0)	0.1 (0.1, 25.2)	0.1 (0.1, 7.6)
IL-5	6.5 (2.9, 26.2)	3.8 (3.5, 24.0)	3.8 (2.4, 10.2)
IL-6	24.3 * (17.5, 74.2)	16.0 (4.4, 52.4)	7.3 (2.2, 21.1)
IL-7	22.6 (9.7, 66.6)	29.3 (8.7, 63.5)	12.6 (5.8, 38.1)
IL-8	1389 (329, 3325)	681 (409, 1867)	400 (209, 1280)
IL-10	5.6 (1.5, 11.8)	4.8 (1.6, 10.9)	2.1 (1.5, 7.1)
IL-12	41.5 (20.3, 114.0)	33.3 (18.4, 113.2)	18.4 (7.7, 52.7)
IL-13	45.5 (20.5, 97.3)	35.3 (28.1, 103.1)	25.8 (21.5, 68.7)
IL-15	16.5 (0.6, 112.9)	19.0 (0.6, 152.1)	3.1 (0.6, 67.9)
IL-17	116.4 (4.0, 303.6)	86.3 (4.0, 312.0)	16.2 (4.0, 201.2)
IP-10	15.2 (7.2, 29.9)	16.4 (9.9, 23.6)	16.4 (6.4, 63.9)
MCP-1	345 (185, 492)	269 (168, 383)	184 (124, 305)
MIG	73.6 (12.0, 231.6)	157.3 (47.8, 239.3)	60.1 (24.9, 164.8)
MIP-1α	41.9 (17.4, 96.6)	82.6 (22.4, 146.7)	26.1 (17.4, 57.0)
MIP-1β	47.1 (21.0, 109.0)	59.1 (38.3, 113.4)	36.5 (14.9, 85.0)
RANTES	29.3 (19.5, 54.8)	31.1 (20.0, 59.2)	32.5 (12.4, 47.8)
TNF-α	5.0 (1.9, 11.3)	11.8 (2.6, 15.5)	2.6 (1.9, 7.1)

Table 5.7 Post steroid trial sputum cytokines.

Data presented as median pg/ml (IQR). *; $p < 0.05$

5.3.9.2 Plasma cytokine responses to oral corticosteroids

The difference between smokers and non smokers for plasma IL-1RA and IL-13 was still present post oral corticosteroids. The difference between smokers and no-smokers for plasma GM-CSF and IL-10 was narrowed as a result of oral corticosteroids and lost statistical significance. However other plasma cytokines changed in response to the oral corticosteroid trial resulting in significant differences becoming evident when smokers and non smokers with asthma were compared (table 5.8):

- IFNα; smokers 29.5 pg/ml (IQR 24.5, 34.3), non smokers 34.3 pg/ml (IQR 31.2, 37.2), p=0.035

- IL-5; smokers 2.2 pg/ml (1.9, 2.2), non smokers 2.5 pg/ml (2.2, 2.5), p=0.006

- IL-7; smokers 7.6 pg/ml (6.8, 14.5), non smokers 15.4 pg/ml (13.5, 17.1), p=0.019

- MIP-1α; smokers 20.8 pg/ml (20.8, 22.4), non smokers 24.1 pg/ml (22.4, 27.3), p=0.016

- MIP-1β; smokers 24.4 pg/ml (21.5, 30.1), non smokers 30.1 pg/ml (27.8, 32.4), p=0.011

- TNFα; smoker 2.1 pg/ml (1.9, 2.5), non smokers 2.7 pg/ml (2.4, 2.9), p=0.007

	Smokers	Non Smokers
Eotaxin	149.2 (97.9, 208.3)	101.1 (82.5, 123.5)
GM-CSF	10.3 (5.1, 11.6)	10.3 (6.6, 18.6)
IFN-α	29.5 * (24.5, 34.3)	34.3 (31.2, 37.2)
IFN-γ	1.0 (0.3, 2.0)	0.5 (0.3, 3.1)
IL-1RA	142.1 * (116.6, 178.5)	178.5 (160.3, 214.9)
IL-1β	12.0 (9.3, 16.1)	13.4 (12.7, 19.4)
IL-2	4.4 † (3.8, 5.3)	5.0 (4.4, 7.2)
IL-2R	176.6 (132.2, 220.3)	195.4 (170.3, 232.6)
IL-4	6.3 (4.8, 9.3)	7.3 (5.3, 11.3)
IL-5	2.2 * (1.9, 2.2)	2.5 (2.2, 2.5)
IL-6	1.4 (1.1, 1.8)	1.6 (1.2, 4.5)
IL-7	7.6 * (6.8, 14.5)	15.4 (13.5, 17.1)
IL-8	4.3 (3.1, 4.8)	4.0 (2.9, 6.1)
IL-10	1.4 (1.4, 1.7)	1.5 (1.5, 2.4)
IL-12	58.1 (39.8, 63.8)	53.2 (47.8, 59.4)
IL-13	19.5 * (17.4, 21.5)	21.5 (19.5, 25.3)
IL-15	1.3 (0.6, 4.0)	2.2 (0.6, 11.9)
IL-17	6.1 (4.0, 39.6)	28.3 (4.0, 38.5)
IP-10	3.9 * (3.2, 5.4)	6.4 (4.3, 9.7)
MCP-1	116.8 (92.1, 181.5)	122.3 (103.7, 192.3)
MIG	12.0 (12, 12)	12.0 (12, 12)
MIP-1α	20.8 * (20.8, 22.4)	24.1 (22.4, 27.3)
MIP-1β	24.4 * (21.5, 30.1)	30.1 (27.8, 32.4)
RANTES	3273 (2888, 4134)	4773 (3390, 5449)
TNF-α	2.1 * (1.9, 2.5)	2.7 (2.4, 2.9)

Table 5.8 Post steroid trial plasma cytokines.

Data presented as median pg/ml (IQR). *; $p < 0.05$

Examination of within group changes demonstrated that a number of plasma cytokines changed in response to oral corticosteroid trial. Smokers with asthma demonstrated an increase in plasma eotaxin (48.5 pg/ml (95% CI 23.6, 73.4), p=0.001) and reduction in:

- IFNγ; -1.5 pg/ml (95%CI -2.7, 0.2), p=0.022

- IL-2R; -88.8 pg/ml (-137.4, -40.1), p=0.001

- IL-4; -3.2 pg/ml (-5.2, -1.3), p=0.003

- IL-5; -0.5 pg/ml (-0.8, -0.2), p=0.003

- IL-7; -5.1 pg/ml (-10.2, -0.0), p=0.049

- IL-8; -1.9 pg/ml (-2.8, -0.9), p=0.001

- IL-12; -7.7 pg/ml (-12.0, -3.4), p=0.002

- IP-10; -7.6 pg/ml (-10.0, -5.2), p<0.001

- MCP-1; -59.2 pg/ml (-109.3, -9.0), p=0.024

- MIP1β; -3.7 pg/ml (-6.5, -0.9), p=0.014

- RANTES; -2207 pg/ml (-3706, -707), p=0.007

Non smokers with asthma also made a number of significant within group changes in response to oral corticosteroid. Plasma eotaxin increased (36.8 pg/ml (95% CI 21.0, 52.6), p<0.001) and reductions were evident in:

- IL-1RA; -71.7 pg/ml (95% CI -123.9, -19.5), p= 0.010

- IL-2R; -54.7 pg/ml (-95.4, -13.9), p=0.012

- IL-4; -2.4 pg/ml (-4.6, -0.3), p=0.027

- IL-8; -1.2 pg/ml (-2.3, -0.1), p=0.033

- IL-12; -20.8 pg/ml (-31.3, -10.4), p=0.001

- IP-10; -5.9 pg/ml (-10.3, -1.6), p=0.011

- RANTES; -2106 pg/ml (-3961, -252), p=0.029

Comparison of the within group changes in plasma cytokines demonstrated that plasma IL-12 was reduced to a greater degree in non-smokers with asthma compared to smokers with asthma in response to the oral corticosteroid trial (non-smokers change -19.0 pg/ml (-26.5, -6.6), smokers -8.9 pg/ml (-11.5, -1.1), p=0.025).

5.3.10   IL-6 high sensitivity ELISA

5.3.10.1   ELISA results

A high sensitivity ELISA was performed to examine the IL-6 sputum supernatant result obtained by Luminex. By this method smokers with asthma again had a higher median concentration of sputum IL-6 compared to non-smokers with asthma at baseline (smokers 14.5 pg/ml (IQR 9.1, 59.9), non-smokers 3.1 pg/ml (IQR 0.4, 6.6), p<0.001) and post oral corticosteroids (smokers 10.0 pg/ml (2.7, 33.5), non-smokers 3.1 pg/ml (0.0, 8.9), p=0.041) (table 5.9 & figure 5.13). Ex-smokers levels of sputum IL-6 appeared to be equivalent to smokers with asthma at baseline. Post oral corticosteroid trial the ex-smokers appeared to have a sputum IL-6 level closer to non-smokers with asthma.

High dose oral corticosteroids did not reduce sputum IL-6 levels in smokers (Change -17.4 pg/ml (95% CI -58.0, 23.1), p=0.376) and non-smokers with asthma (Change 7.1 pg/ml (-9.7, 23.1), p=0.383).

	Smokers	**Ex-smokers**	**Non-Smokers**
Pre-steroid IL-6 (pg/ml)	14.5 [‡] (9.1, 59.9)	11.6 (4.3, 50.3)	3.1 (0.4, 6.6)
Post steroid IL-6 (pg/ml)	10.0 [*] (2.7, 33.5)	2.9 (0.4, 23.9)	3.1 (0.0, 8.9)

Table 5.9 Pre and post sputum IL-6 levels measured by high sensitivity ELISA.

Data presented as median (IQR). *; p<0.05, ‡; p<0.001
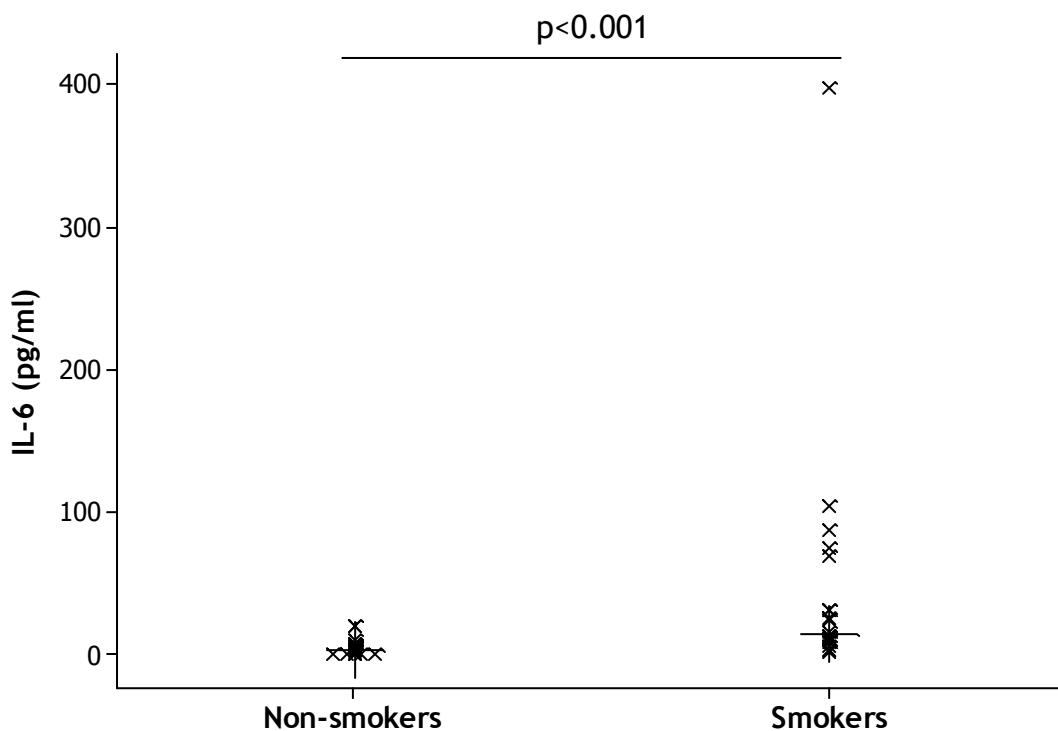
Figure 5.13 Individual plot of IL-6 levels obtained by high sensitivity ELISA in non-smokers with asthma and smokers with asthma.

Data presented as individual points with median.

5.3.10.2   Comparison of Luminex and ELISA results

When the two methods were compared there was evidence of a strong correlation between the sputum IL-6 results obtained by Luminex and high sensitivity ELISA:

- Smokers; r = 0.84, p<0.001 (figure 5.14)

- Ex-smokers; r = 0.89, p=0.001

- Non-smokers; r = 0.84, p<0.001
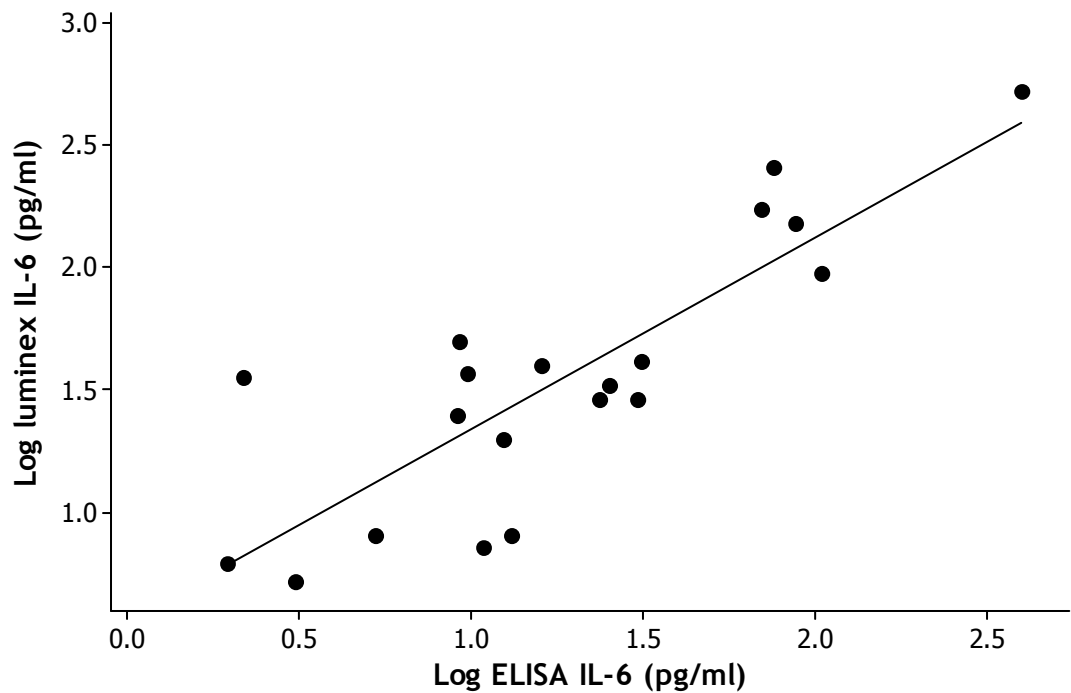
Figure 5.14 Correlation between Log Luminex IL-6 and Log ELISA IL-6 results for smokers with asthma.

5.4 Discussion

Smokers with asthma fail to gain the expected benefits from both inhaled and oral corticosteroids (5-10, 22-24). This reduced response is associated with an accelerated decline in lung function (15, 21), increased emergency department visits (16, 17) and increased severity of asthma symptoms compared to non-smoking subjects with asthma (11, 12). The route by which smoking alters the corticosteroid responsiveness of smokers with asthma is currently unclear with several mechanisms proposed (32, 40). However corticosteroids are recognised to reduce pro-inflammatory cytokine levels with associated beneficial effects in asthma, and an altered cytokine environment can induce corticosteroid insensitivity in T lymphocytes *in-vitro* (66-69). An altered cytokine environment has also been observed in non-smoking corticosteroid resistant asthmatics (64, 65). Therefore altered cytokine profiles may be relevant to the development of reduced corticosteroid sensitivity in smokers with asthma.

An important issue to consider is that multiple significance tests were performed to generate the results presented in this chapter. At a significance level of 5%

the odds of a false positive result is as high as one test in twenty. Therefore a number of false positive differences are possibly contained in the presented data. Correction through the use of the Bonferonni adjustment resulted in all baseline sputum (save IL-6) and plasma cytokines losing statistical significance. However the application of such methods can be viewed as a dubious way to deal with data gathered from a small exploratory study as strong and mechanistically significant observations will be dismissed as a result. Examining multiple cytokines in an unbiased fashion is also more likely to provide significant insights into the causes of corticosteroid resistance as cytokines do not work in isolation and determining patterns of alteration are important. The large number of significantly altered cytokines discovered using the multiplex approach suggests that smokers with asthma do have a significantly different cytokine profile compared to non smokers with asthma. Future adequately powered studies should examine these findings.

In this cross sectional study, smokers with asthma failed to gain significant improvements in lung function during an oral corticosteroid trial. This reduced response reflects and confirms previous research in smokers with asthma (23, 24). Smokers and non-smokers with asthma displayed equivalent sputum differential counts at baseline. The finding of a lack of corticosteroid response in this group of smokers with asthma despite the absence of sputum neutrophilia is interesting and suggests that the resistance in the recruited subjects is not due neutrophilic inflammation. The contrast in lung function response between the smokers and non smokers with asthma was associated with a number of differences in sputum and plasma cytokine levels. Sputum supernatants obtained from smokers with asthma demonstrated increased levels of IL-2, 4, 6, 7 & IFNγ and a borderline increased level of IL-12 and 17.

Significant differences were present at baseline for inhaled corticosteroid dose and ACQ score between the smokers and non smokers with asthma. Previous research has demonstrated that smokers with asthma have higher ACQ scores despite matched lung function measures (12) and the increased symptoms in this group are likely to have led to the prescription of increased doses of inhaled corticosteroids. To examine the effect of this difference an adjustment of the sputum cytokine results for differences in baseline inhaled corticosteroid dose was performed. The expectation was that this adjustment would remove any

false associations. The adjustment did not reduce the significant differences in IL-2, 4, 6, 7 and IFNγ between smokers and non-smokers with asthma, resulted in multiple additional cytokine differences becoming evident and strengthened the difference present in sputum IL-12 and 17. The presence of increased levels of several cytokines in induced sputum supernatant from smokers with asthma suggests that this group display an altered and increased level of airway inflammation compared to non smokers with asthma with similar lung function. Are the increases in sputum cytokines demonstrated in this study responsible for the corticosteroid resistance seen in smokers with asthma?

The largest difference in sputum cytokines between smokers and non-smokers was for IL-6 and this is the only cytokine difference that would survive p value adjustment. The presence of a significant difference between smokers and non-smokers was also subsequently confirmed by high sensitivity ELISA. This increase in sputum IL-6 was resistant to oral corticosteroids and is a novel observation in smokers with asthma. IL-6 is a pleiotropic pro-inflammatory cytokine which sits at the junction between the innate and adaptive immune response and has an important role in Th-17 differentiation (278). Previous research has demonstrated an inverse correlation between IL-6 and lung function in COPD (279). IL-6 is also upregulated in subjects with COPD (280) and in both COPD and asthma during exacerbations (281, 282). Bronchoalveolar lavage samples from corticosteroid resistant non-smokers with asthma have been demonstrated to contain increased levels of IL-6 (65). The finding in this study that IL-6 is increased in smokers with asthma and is unresponsive to high dose oral corticosteroids suggests that sputum IL-6 may play an important role in the development of corticosteroid insensitivity in smokers with asthma. IL-6 signals via signal transducer and activator of transcription-3 (STAT3) resulting in increased gene transcription and activation of NF-κB (283-285). Therefore IL-6 acting via STAT3 and other gp130 coupled pathways may induce persistent corticosteroid insensitive inflammation in smokers with asthma akin to a persistent viral infection. This finding is of interest in the light of the recent development of IL-6 receptor blockers for the treatment of inflammatory conditions. IL-6 receptor blockade may therefore represent a useful approach in smokers with poorly controlled asthma.

Previous research has demonstrated increased expression in IL-2 and 4 in cells obtained by bronchoalveolar lavage from corticosteroid resistant non-smokers with asthma (64). This finding was confirmed and extended by the demonstration that the combination of IL-2 and 4 can induce a corticosteroid resistant state in peripheral blood T lymphocytes *in-vitro* (66-69). The effects of the combination of IL-2 and IL-4 can be overcome by the simultaneous co-administration of IFNγ *in-vitro* (69) and corticosteroid responsive asthmatics demonstrate an increase in IFNγ expressing cells following treatment with oral prednisolone (64). This alteration in IFNγ expression mirrors the narrowing of the difference in IFNγ concentration following corticosteroids observed in this study. The finding that detectable levels of IL-2 and 4 are present in the sputum supernatant of corticosteroid resistant smokers with asthma is intriguing and the finding of increased IFNγ at baseline may be an indication of an intrinsic attempt to overcome the effects of raised IL-2 & 4. However previous attempts at measurement of IL-4 in *ex-vivo* samples have been fraught with difficulty. Given the low levels of IL-2 and 4 detected in the samples in this study any conclusions for these cytokines must be cautious. Further work examining BAL cytokine levels of IL-2, 4 and IFNγ tied to clinical characterisation and examination of corticosteroid responses of *ex-vivo* samples is required before firm conclusions can be drawn.

IFNγ is regarded as a characteristic Th1 cytokine with a role for the induction of various cytokines. Sputum IL-6 can also be regarded in this light. Can the alteration in sputum cytokines in smokers with asthma be a result of smoking producing a skewing of asthma from a Th2 to a Th1 phenotype? This could be possible. COPD is associated with increased pulmonary and systemic expression of IL-6 (286-288) and cells expressing IFNγ in bronchial biopsies (289, 290). IL-12, which was on the threshold of statistical significance, was also raised in the smokers with asthma and is important for the induction of IFNγ expression (291). Non smoking subjects with severe asthma who fail to gain the expected benefits from corticosteroids express increased levels of IFNγ (275, 292) and IFNγ is known to both promote the expression of Th1 cytokines and suppress those associated with Th2 environments (293). Given the presence of the common environmental factor of smoking it is tempting to extrapolate that smokers with asthma have an alteration in their inflammatory response from the Th2 response

characteristic of allergic asthma to the Th1 inflammation present in COPD. Smokers with asthma and subjects with COPD both display reduced corticosteroid responsiveness and accelerated lung function decline and if the inflammation present in subjects with COPD and smokers with asthma shares a common cytokine profile then therapies designed for COPD may also prove to be effective in smokers with asthma.

IL-7 is essential for the development of T and B lymphocytes and may play a role in dendritic cell survival (294). The finding of increased sputum supernatant IL-7 in smokers with asthma is novel and may reflect previous work which identified a reduction in bronchial biopsy B-lymphocyte and dendritic cell numbers in smokers with asthma (62). Raised sputum cytokine IL-7 levels may reflect an attempt at restoration of airway B lymphocyte numbers. No differences were evident in sputum lymphocyte proportions between smokers and non smokers with asthma. However sputum lymphocyte numbers are usually very low so it will be difficult to detect a difference in airway T lymphocyte numbers even if this is present. Further investigation of the role of IL-7 and IL-7 homologues such as Thymic Stromal Lymphopoetin in the control of airway T and B lymphocytes and airway dendritic cells in smokers with asthma should be considered.

The lack of a difference for sputum IL-8 when smokers and non-smokers with asthma were compared contrasts with previous work in smokers with asthma (41). However direct comparison with the previous study is difficult as the smokers with asthma in the prior study were not prescribed inhaled corticosteroids. The mean pack year history of the smokers with asthma recruited to this trial were higher than the previous study and the standard deviations were equivalent. Therefore the narrowing of the difference between the two groups is not due to lower pack year histories in the recruited group and appears to be due to the existence of a group with lower levels of IL-8 reflected in the lower quartile which overlaps with the non-smokers with asthma. This could reflect alteration in smoking habits, with the current group of smokers with asthma smoking less per day. An alternative explanation is that the lack of a difference may reflect technical differences as the assay employed for the detection of IL-8 was different. The previous study employed an ELISA method and the current study used Luminex and this may be partially responsible for to the lack of difference in this study. However correlations were still present

between sputum IL-8 and a number of clinical characteristics corroborating the previous study and the importance of this cytokine in smoking related pulmonary responses.

Overall the data presented in this chapter, allied to previous work demonstrating increased sputum IL-8 (41) and reduced IL-18 (42) in smokers with asthma, suggests that the inflammation present in this group is different from non-smokers with asthma and represents a unique inflammatory phenotype. Unfortunately it is not possible to be certain as to the cellular sources responsible for the altered sputum cytokines detected in smokers with asthma as samples were not processed for immunocytochemistry. A number of cells could be responsible for the increased cytokines and several cell types may be contributing. For example, macrophages (65, 70), bronchial epithelial cells (295) and T lymphocytes can all produce IL-6. It is difficult to conclude which of these or indeed if all cell types are responsible for the differences detected between smokers and non smokers with asthma. Future work needs to address this issue.

The inclusion of a small group of ex-smokers with asthma allowed for a limited examination of the persistent effect of smoking on asthma. A previous study which examined oral corticosteroid responses in smokers with asthma suggested that ex-smokers with asthma have a heterogeneous response to corticosteroids (23). A similar response was evident in the group of ex-smokers recruited for this study despite the average duration of smoking cessation being over seven years. The ex-smokers with asthma had similarities in their cytokine profile to both smokers and non-smokers with asthma. Given the heterogeneous response of the ex-smokers with asthma to oral corticosteroids consideration should be given to an adequately powered study to determine the alterations in cytokine profiles following smoking cessation that predict the restoration of corticosteroid response.

Examination of systemic cytokine profiles using peripheral blood samples also revealed differences between smokers and non smokers with asthma at baseline. However a different cytokine profile emerged with smokers with asthma generally expressing lower levels of plasma cytokines than non smokers with asthma. Smokers with asthma had significantly lower levels of plasma IL-1RA, 10, 13 and GM-CSF and a borderline reduced level of IL-12 compared to non

smokers with asthma. No difference was evident in plasma IL-2, 4, 6, 7 or IFNγ. This reduction in plasma cytokines suggests that smokers with asthma, in contrast to COPD, do not suffer from a generalised systemic inflammation due to an 'overspill' of pulmonary inflammation. The presence of increased sputum supernatant IL-6 but similar peripheral IL-6 levels in smokers with asthma also suggests that smokers with asthma display a different phenotype to both subjects with COPD and non smokers with asthma. However high sensitivity CRP concentrations were not determined in this study and need to be performed to allow true comparison with the previous work in subjects with COPD. The disparity between the sputum and systemic cytokine levels means that future studies should concentrate on measurement of cytokines from airway samples in preference to peripheral blood. Future research should compare smokers with asthma to non smokers with asthma and subjects with COPD to determine the similarities and differences in inflammatory system activity between the groups.

The cytokine profiles post corticosteroid trial revealed a number of interesting findings. Smokers with asthma, despite failing to respond clinically, did reduce a number of sputum supernatant cytokines. Non-smokers with asthma also reduced a number of sputum cytokines in association with an improvement in lung function. Which cytokines responded differently when the two groups were compared? Post corticosteroid sputum levels of IL-1RA, 6 and Eotaxin were significantly higher in the smokers with asthma. Sputum IL-2, 4 and IFNγ did not significantly change in either smokers or non smoker with asthma in response to oral corticosteroids but statistical significance was lost. This may reflect an increase in IL-2, 4 & IFNγ in non smokers with asthma, a reduction in the cytokines in smokers with asthma or reflect variability in the assay. However comparison of within group changes did not reveal any cytokines that behaved differently in the non smokers with asthma and therefore the baseline comparisons are likely to provide the greatest insights. The failure of sputum IL-6 to reduce in response to high dose oral corticosteroids again suggests that this cytokine is associated with the mechanism(s) responsible for the reduced lung function response in smokers with asthma. Further studies are required to examine this cytokine in characterised smokers and non smokers with asthma.

5.5 Conclusion

Smokers with asthma display alterations of both sputum supernatant and peripheral cytokine profiles that are associated with reduced response to oral corticosteroids. The increase in sputum IL-6 displayed by smokers with asthma which is resistant to oral corticosteroids may reflect increased NF-κB activation via increased STAT3 leading to corticosteroid resistant inflammation. The dissociation between peripheral and sputum cytokine profiles demonstrates that direct sampling in smokers with asthma may be crucial to the determination of the causes of the altered corticosteroid response in this group. Future studies should endeavour to examine cytokine profiles in smokers with asthma with comparison to subjects with COPD using bronchoscopic sampling as this will allow reference to the responsible airway cell populations and allow further dissection of the inflammatory processes in this group.

6 Relevance of HDAC activity to corticosteroid response in smokers with asthma

6.1 Introduction

Smokers with asthma exhibit an impaired response to both inhaled and oral corticosteroids (5-10, 22-24). Previous research has demonstrated that smokers with asthma also suffer increased symptoms (11, 12), an accelerated decline in lung function (15, 21) and increased emergency department visits for asthma (16, 17) compared to matched non-smoking asthmatics.

The prevalence of smoking in asthma reflects that of the general population and therefore smokers with asthma represent a large group of patients with poorly controlled disease (13). Smoking cessation is the obvious target for both health practitioners and smokers with asthma and this approach has been demonstrated to be an effective therapy in this group (49). However as sustained quitting rates are low either improvements on current treatments or alternative therapies are required for individuals with asthma who continue to smoke. Development of new treatments requires understanding of the alteration in the phenotype of asthma induced by smoking and its relationship to treatment response.

The discipline of epigenetics has revealed a mechanism that may be of relevance to the reduced response to corticosteroids observed in smokers with asthma. Epigenetics examines the effect of post-translational covalent modifications of chromatin on the control of gene expression. An *in-vitro* model of inflammation has demonstrated that approximately half of the immunosuppressant activity of corticosteroids is produced via the removal of acetyl groups from DNA associated histone proteins (98). The removal of acetyl groups from histone proteins at areas of active transcription results in a conformational change in chromatin in the targeted area leading to cessation of active transcription (81). Cigarette smoke has been demonstrated to reduce HDAC activity *in-vitro* (92) and a reduction in HDAC activity in smokers with asthma could explain their relative corticosteroid insensitivity.

Therefore this cross sectional study was designed to test the hypothesis that smokers with asthma have a reduced level of HDAC activity compared to non-smokers with asthma and that this is associated with reduced corticosteroid responsiveness.

6.2 Methods

6.2.1 Subjects

Subject characteristics, inclusion/exclusion criteria and recruitment methods were as described in the general methods chapter. All subjects provided informed consent and the study was approved by the West Glasgow Ethics Committee.

6.2.2 Study design

A full description of the study is provided in the general methods chapter. In brief, the subjects were recruited to a cross-sectional study with unblinded use of oral dexamethasone to determine corticosteroid sensitivity. The baseline visit consisted of a number of assessments including sputum and blood for macrophage/monocyte HDAC activity assessment and lung function measurement by spirometry. At the completion of the corticosteroid trial subjects were re-assessed within 24 hours of their last dose and repeated spirometry, venesection and sputum induction for HDAC activity assessment.

6.2.3 Measurements

A full description of the measurements is provided in the general methods chapter. Lung function assessments conformed to consensus guidelines (246). Sputum induction and processing and blood processing for HDAC measurement was performed as discussed in the general methods chapter. Assay variability testing was not performed for the HDAC test utilised in this thesis.

6.2.4 Statistical analysis

Parametric data was examined using paired t-testing or 2 sided t-testing and non-parametric data with Wilcoxon-Mann-Whitney testing. Comparisons were between smokers and non-smokers with asthma. Ex-smokers were not included in formal comparison analyses due to the small number of subjects in this group. Correlations were performed predominately with Spearman Ranks and supplementary calculations with Pearson's (indicated by ρ for Spearman and r for Pearson when presented). Statistical analysis was performed using Minitab 15 (Minitab Inc. State College, PA, USA) and SAS v 9.1 (TS1M3) for Windows (SAS Institute Inc., NC, USA). α was set at 0.05. No adjustments were performed for multiple comparisons.

6.3 Results

Sputum samples suitable for measurement of HDAC activity were obtained from 18 non-smokers, 9 ex-smokers and 18 smokers with asthma at baseline and from 14 non-smokers, 9 ex-smokers and 14 smokers with asthma at completion of the corticosteroid trial. Suitable baseline blood samples were obtained from 20 non-smokers, 9 ex-smokers and 22 smokers with asthma and from 17 non-smokers, 9 ex-smokers and 18 smokers with asthma at completion of the corticosteroid trial.

6.3.1 Baseline comparisons

The baseline characteristics are discussed in detail in chapter 5 sections 5.3.1 to 5.3.4. A brief review of the relevant findings will be presented here.

6.3.1.1 Clinical characteristics

Subjects were well matched for clinical variables at baseline. Smokers with asthma were taking higher levels of inhaled corticosteroids and had higher ACQ scores compared to non-smokers with asthma.

6.3.1.2 Lung function measurements

Smokers and non-smokers with asthma were well matched for lung function measures at baseline, although non-smokers with asthma demonstrated a greater degree of bronchodilator response compared to smokers with asthma.

6.3.1.3 Baseline sputum characteristics

No differences were present between smokers and non smokers when baseline sputum profiles were compared. There was an absence of eosinophilia in both smokers and non-smokers with asthma.

6.3.2 Corticosteroid response

Non-smokers with asthma made a significant lung function response to oral corticosteroids. This was in contrast to smokers and ex-smokers with asthma who failed to make a significant within group response to oral corticosteroids. No difference was evident when between groups responses were compared.

6.3.3 Change in clinical characteristics and sputum profile

Non-smokers with asthma demonstrated a reduction in ACQ score in response to the oral corticosteroid trial in contrast to smokers with asthma. No significant differences in the response of ACQ score and sputum to dexamethasone were detectable between the smokers and non smokers with asthma.

6.3.4 Baseline HDAC activity

6.3.4.1 Baseline sputum macrophage HDAC activity

No differences were detectable between smokers and non smokers with asthma in baseline sputum HDAC activity levels (smokers 92.8 µmol/l/10^{-6} cells (IQR 7.2, 277.8), non smokers 82.7 µmol/l/10^{-6} cells (34.3, 150.5) p=0.960) (table 6.1 and figure 6.1). Ex-smokers had a similar sputum HDAC activity level (55.4 µmol/l/10^{-6} cells) to smokers and non smokers with asthma.
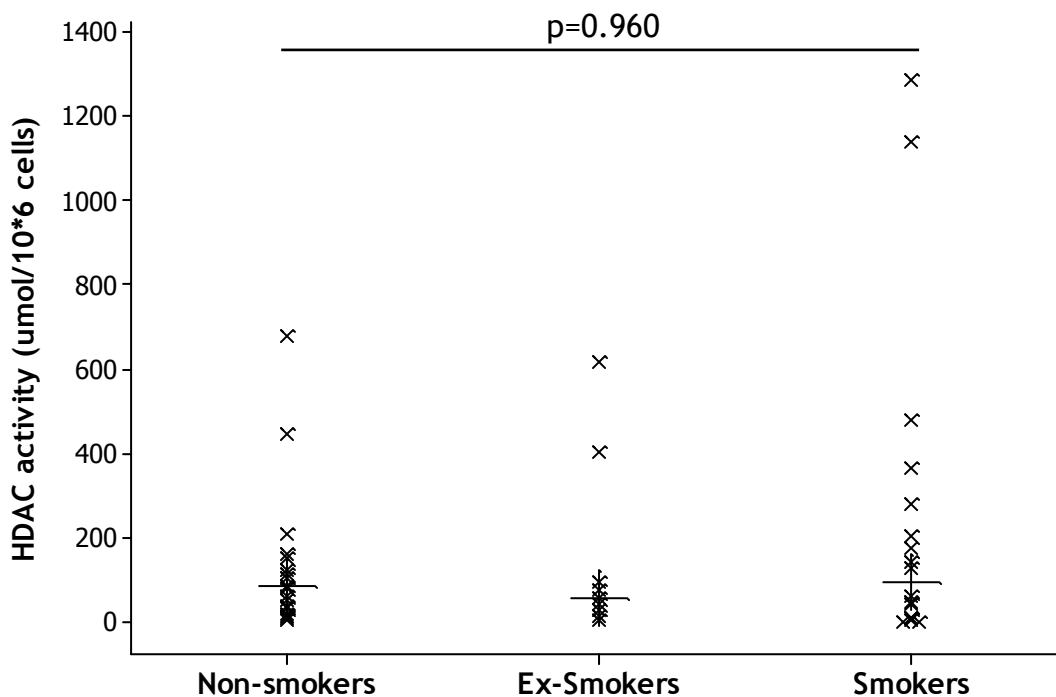
Figure 6.1 Baseline sputum HDAC activity in non-smokers, ex-smokers and smokers with asthma.

6.3.4.2 Baseline blood monocyte HDAC activity

Baseline blood HDAC activity was equivalent in both smokers and non-smokers with asthma (smokers 1.63 µmol/l/10^{-6} cells (IQR 1.19, 3.10) non smokers 1.99 µmol/l/10^{-6} cells (1.52, 3.84), p=0.180) (table 6.1 and figure 6.2). Ex-smokers with asthma blood HDAC activity levels were equivalent to smokers and non smokers with asthma.

	Smokers	**Ex-smokers**	**Non smokers**
Baseline sputum HDAC activity µmol/10*6	92.8 (7.1, 299.2)	55.4 (20.7, 247.7)	82.7 (30.2, 153.1)
Baseline blood HDAC activity µmol/10*6	1.63 (1.19, 3.10)	2.51 (1.81, 3.65)	1.99 (1.52, 3.84)

Table 6.1 Comparison of HDAC activity across groups for sputum and blood.

Data presented as median (IQR).
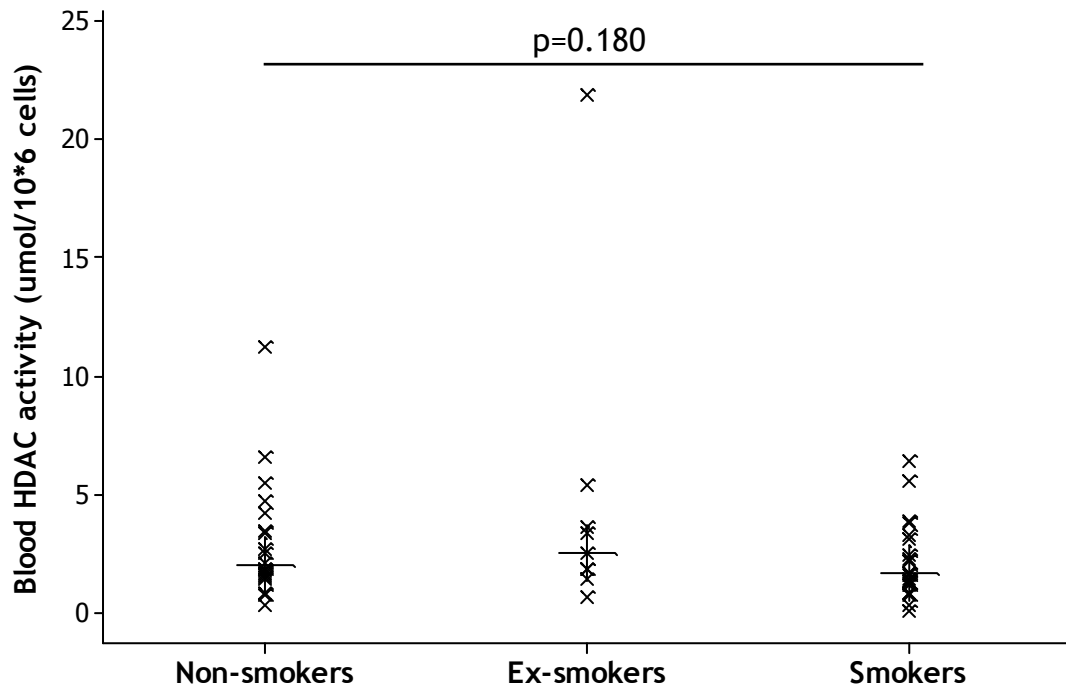
Figure 6.2 Baseline blood HDAC activity in non-smokers, ex-smokers and smokers with asthma.

6.3.4.3 Relationship of sputum HDAC activity to lung function response to dexamethasone

The baseline sputum HDAC activity of smokers with asthma did not predict FEV_1 response to dexamethasone. When examined using a 15% improvement in FEV_1 as the discriminator of corticosteroid responsiveness, the average HDAC activity of the two corticosteroid responsive smokers was 24.1 µmol/l/10^{-6} cells. Sputum HDAC activity of the unresponsive group was 135.3 µmol/l/10^{-6} cells (IQR 7.2, 342.1). When the same comparison was made in the non-smokers with asthma no difference was obvious between the subjects who responded and those that failed to improve (responders 103.2 µmol/l/10^{-6} cells (2.3, 150.5), non responders 82.7 µmol/l/10^{-6} cells (30.2, 172.3) p=0.753).

No correlation was evident between baseline sputum HDAC activity and FEV_1 response to oral corticosteroids (ρ=-0.10 (95% CI -0.41, 0.22), p=0.519) (figure 6.3). When examined as separate groups no correlations were evident between baseline sputum HDAC activity and FEV_1 response to corticosteroids:

- Smokers with asthma; ρ=-0.14 (-0.59, 0.38), p=0.601

- Non-smokers with asthma; ρ= -0.08 (-0.59, 0.47), p=0.764

- Ex-smokers with asthma; ρ= 0.00 (-0.70, 0.70), p=1.000
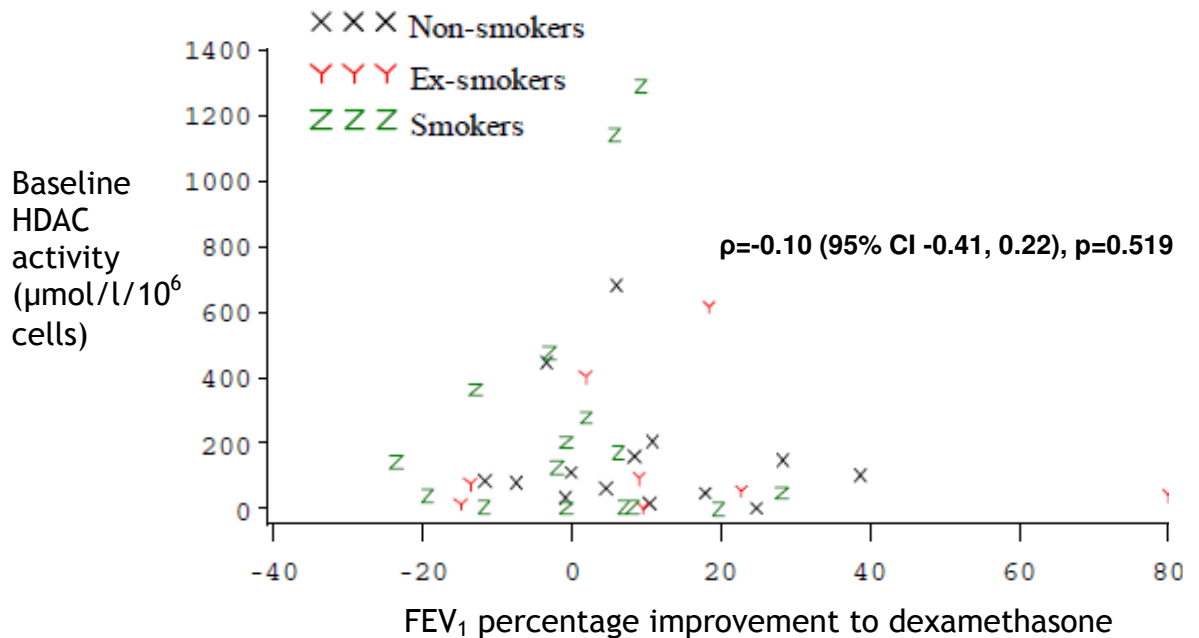
Figure 6.3 Relationship between FEV$_1$ response to dexamethasone and baseline sputum macrophage HDAC activity.

6.3.4.4 Relationship of blood HDAC activity to lung function response to dexamethasone

Baseline blood HDAC activity similarly failed to predict lung function response in smokers with asthma (>15% response mean activity 1.6 μmol/l/10^{-6} cells, <15% response 1.7 μmol/l/10^{-6} cells (IQR 0.9, 3.2)) and non-smokers with asthma (<15% response 1.9 μmol/l/10^{-6} (1.3, 3.8), >15% response 1.5 μmol/l/10^{-6} (1.4, 4.0)).

To allow a further examination of the available data, correlations were performed between the lung function changes for all subjects and HDAC activity. No relationship was evident between the FEV$_1$ response across all groups and baseline blood HDAC activity (ρ= -0.08 (95% CI -0.36, 0.21) p=0.611) (figure 6.4).

When examined according to smoking history no positive correlations were evident between lung function response and blood HDAC activity:

- Smokers with asthma; ρ= -0.37 (95% CI -0.70, 0.08), p=0.097

- Non-smokers with asthma; ρ= -0.05 (-0.54, 0.45), p=0.824

- Ex-smokers with asthma; ρ= 0.38 (-0.38, 0.83), p=0.299
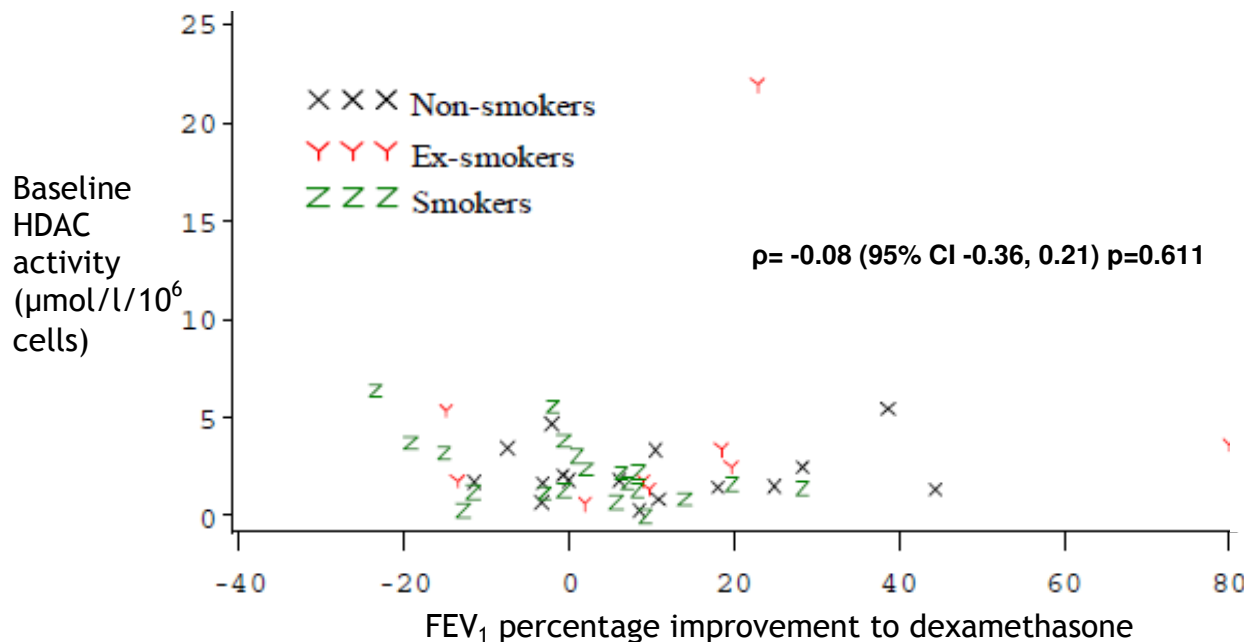
Figure 6.4 Relationship between FEV$_1$ response to dexamethasone and baseline blood monocyte HDAC activity.

6.3.4.5 Correlation between sputum HDAC and blood HDAC activity

No relationship was evident between blood and sputum HDAC activity at baseline (Pearson correlation; r= -0.22, p = 0.163) (figure 6.5)
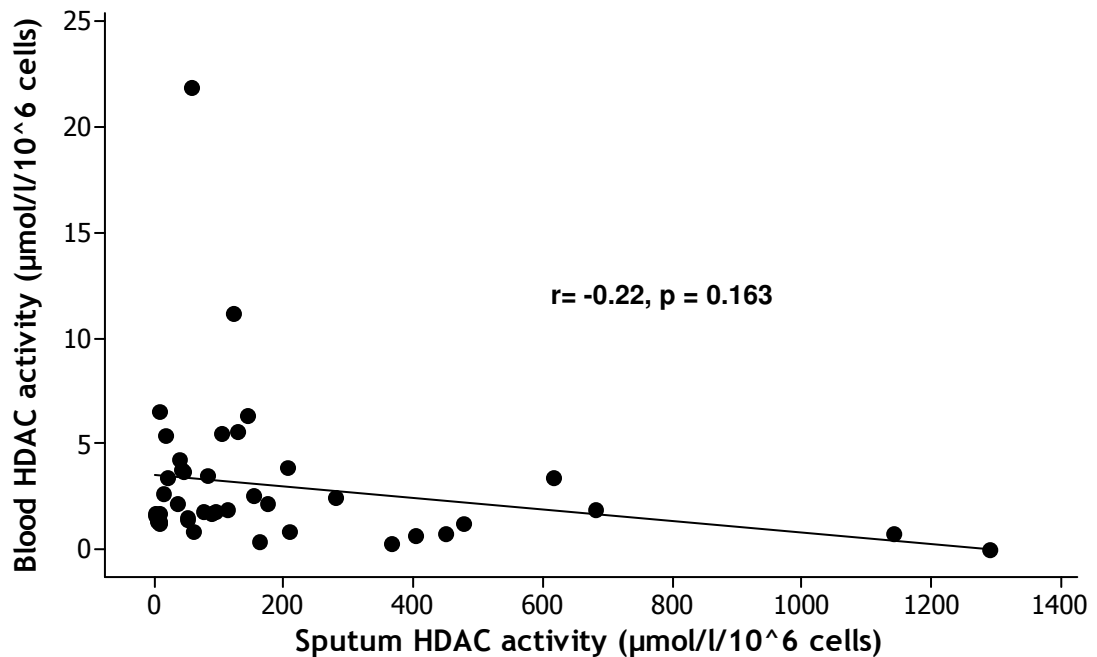
Figure 6.5 Scatterplot of baseline blood and sputum HDAC activity (with regression).

6.3.5 Change in HDAC activity in response to dexamethasone

6.3.5.1 Change in sputum HDAC activity

No change was detectable in sputum HDAC activity within the smoker, ex-smoker or non smoker groups. Post dexamethasone sputum HDAC activity in smokers with asthma was equivalent to non-smokers with asthma (smokers HDAC activity 98.3 µmol/l/10^{-6} (IQR 23.9, 558.5), non smokers 33.8 µmol/l/10^{-6} (12.9, 128.6), p=0.220). The change in sputum HDAC activity in response to dexamethasone was also equal (smokers change 53.2 µmol/l/10^{-6}(6.2, 594.4), non-smokers -72.2 µmol/l/10^{-6} (-137.2, 23.7), p=0.120). Ex-smokers demonstrated an increase in HDAC activity of a similar magnitude to smokers with asthma (60.1 µmol/l/10^{-6} (-162.5, -424.6)).

6.3.5.2 Change in blood HDAC activity

No change was detectable in blood HDAC activity in response to dexamethasone in non smokers (HDAC within group change 0.82 µmol/l/10^{-6} (95% CI -0.98, 2.61), p=0.347) and ex-smokers with asthma (-1.83 µmol/l/10^{-6} (-7.03, 3.37), p=0.440).

Smokers with asthma demonstrated a trend to an increase in blood HDAC activity in response to dexamethasone when within group response was examined (7.02 μmol/l/10^{-6} (-0.38, 14.41), p=0.061).

Post corticosteroid blood HDAC activity levels were significantly higher in smokers with asthma compared to non smokers with asthma (smokers 3.36 μmol/l/10^{-6} (2.0, 10.7), non-smokers 1.86 μmol/l/10^{-6} (1.1, 3.2), p=0.022). A trend to significance was evident when changes in blood HDAC activity in response to dexamethasone in smokers and non smokers with asthma were compared (smokers 2.46 μmol/l/10^{-6} (-0.1, 7.2), non-smokers 0.52 μmol/l/10^{-6} (-0.5, 1.4), p=0.074). Ex-smokers with asthma displayed similar levels of blood HDAC activity to smokers post oral corticosteroid trial (ex-smokers 2.50 μmol/l/10^{-6} (2.0, 5.2)).

No relationship was evident between change in blood HDAC activity and lung function response when all subjects were included (ρ= -0.18 (95% CI -0.5, 0.1) p=0.246). When examined as individual groups no correlations were evident between the change in blood HDAC activity and FEV$_1$ response in smokers (ρ= 0.24 (-0.3, 0.6), p=0.329) or non smokers with asthma (ρ= -0.01 (-0.5, 0.5), p=0.958). However a strong and highly significant correlation was found between change in blood HDAC activity and lung function response in ex-smokers with asthma (ρ= -0.94 (-1.0, -0.7), p<0.001) (figure 6.6).
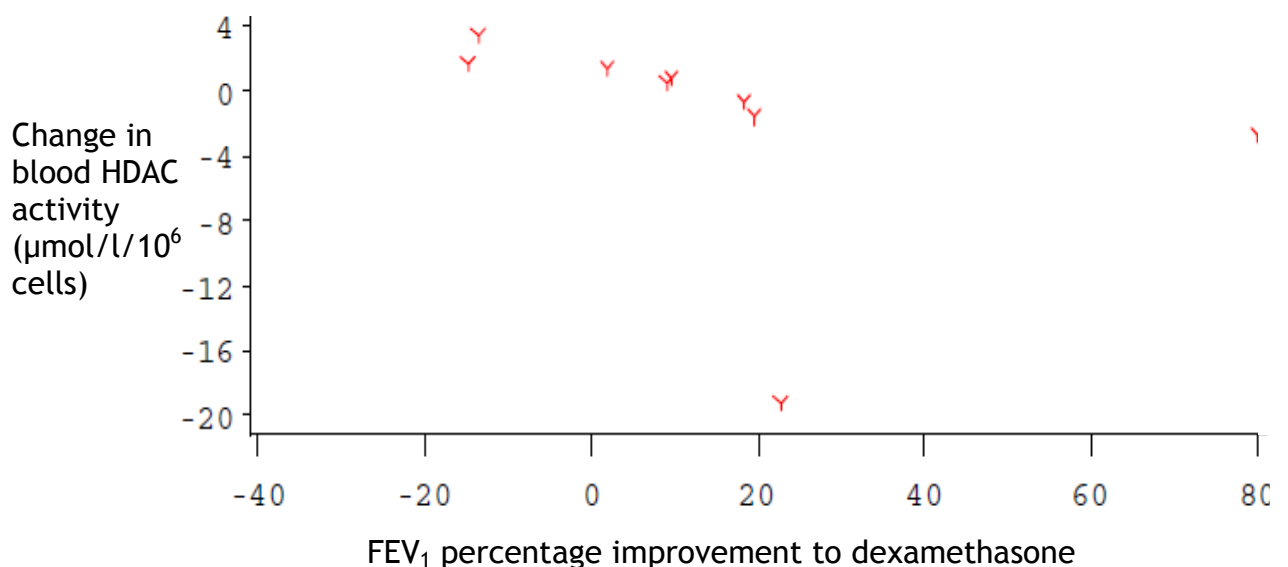
Figure 6.6. Relationship between change in blood HDAC activity and change in FEV$_1$ in ex-smokers with asthma

6.4 Discussion

Smokers with asthma display a reduced response to corticosteroids (5-10, 22-24). The cause (or causes) of this reduced response is currently unknown. The purpose of this exploratory study was to determine HDAC activity levels in this sub group of asthma in comparison to non-smokers with asthma and its relationship to corticosteroid response. Previous research carried out *in-vitro* (103), in subjects with COPD (86, 87) and non-smoking subjects with asthma (84) suggests that reduced HDAC activity is of relevance to corticosteroid responsiveness. Smoking has been demonstrated to reduce HDAC activity *in-vitro* (92) and therefore reduced HDAC activity was expected in smokers with asthma. However this study has provided no evidence to support the hypothesis that HDAC activity is suppressed in either sputum macrophages or blood borne monocytes in smokers with asthma. This was combined with a lack of correlation between lung function response to corticosteroids and HDAC activity.

What can explain this discrepancy? An obvious conclusion is that smokers with asthma do not have a reduced level of HDAC activity and therefore altered HDAC activity does not explain the differences in response to corticosteroid displayed by smokers with asthma. However there are a number of technical issues to consider. The previous research examining the relevance of HDAC activity to corticosteroid response has been carried out in cell lines or cells obtained by bronchoalveolar lavage. Induced sputum has previously been demonstrated to provide different information compared to samples obtained by bronchoalveolar lavage (182) and induced sputum samples the central airways in contrast to bronchoalveolar lavage which obtains samples from smaller airways (183). Therefore a possible explanation that needs to be considered is that the macrophages obtained are of a different phenotype and hence HDAC activity compared to those that exist in the periphery of the lungs. Another consideration is that there was no step to allow for inspection of the cells selected for the HDAC assay. Therefore there is the possibility that contamination with non-viable cells and neutrophils may have affected the results and led to low levels of HDAC activity and lack of a difference between smokers and non-smokers with asthma.

The samples obtained for this study were processed at a research centre with an interest in the examination of HDAC activity in airways disease. Based on previous research using induced sputum, a new technique (in-cell assay) was developed for assessment of HDAC activity to allow for its determination in samples of lower cell numbers. Therefore as this study is the first to use this technique in the comparison of subjects with asthma with differing smoking histories we may be observing a flaw in the technique. Despite best efforts in validation the technique may be too insensitive to detect differences that are present between smokers and non smokers with asthma. An additional consideration is that the absence of a difference in HDAC activity between smokers and non smokers with asthma, if correct, may mask important differences in HDAC containing enzyme isoforms and subsequent alteration in substrate target levels of acetylation. The simple approach taken in this study to address differences in overall level of HDAC activity did not address these issues. Future studies should consider examination of HDAC isoforms and targets of HDAC containing complexes to determine which are important in determining a subject's response to corticosteroids.

An additional weakness of this study is that no attempt was made to assess histone acetyl transferase (HAT) activity. The epigenetic response to corticosteroids can be viewed as an alteration in the balance in HDAC/HAT activity. Previous research has identified HAT activity to be elevated in alveolar macrophages obtained from non-smoking corticosteroid naive subjects with asthma (84). Smokers with asthma may display large increases in HAT activity and this increased HAT activity may be insensitive to corticosteroid treatment. The balance between HDAC and HAT activity that exists in smokers with asthma needs to be addressed in future studies comparing smokers with asthma to non-smokers with asthma and normal subjects.

The absence of correlation between sputum macrophage HDAC activity and lung function change in response to corticosteroid is surprising. However the within group response of the non-smokers with asthma subjects to treatment with oral corticosteroids was slightly smaller than expected. This may reflect that most of the non-smoking subjects with asthma were already treated with moderate doses of inhaled corticosteroids and therefore may not have been able to demonstrate large increases in lung function. Future studies in this area may

need to consider examining groups divided by treatment level and symptom intensity using a system such as the GINA categorisation.

A previous trial has demonstrated that non smokers with asthma do not have altered levels of HDAC activity in peripheral blood compared to normal subjects and that peripheral blood HDAC activity increases in non smokers with asthma in response to an oral corticosteroid trial (84). The presented data demonstrates that smokers with asthma also make a significant increase in peripheral blood monocyte HDAC activity in response to corticosteroids. Ex-smokers with asthma, a proportion of whom were able to demonstrate a good response to oral corticosteroids, demonstrated a highly correlated and significant relationship between lung function improvement and change in blood HDAC activity. However this relationship was reciprocal and currently defies explanation. Previous work in COPD has found that ex-smokers with COPD display differing HDAC responses to treatment compared to smokers with COPD (296) and this finding in ex-smokers with asthma may be a manifestation of an altered inflammatory phenotype that exists in ex-smokers. However there is also the possibility that this result is a type 1 error due to multiple comparisons so further examination of this relationship should be considered before any firm conclusions are drawn.

6.5 Conclusions

This exploratory cross sectional study has demonstrated that smokers with asthma have levels of sputum macrophage HDAC activity comparable to non smokers with asthma. This study has not confirmed a role for reduced HDAC activity in smokers with asthma but should be regarded as inconclusive at present. This finding is surprising but requires further examination and confirmation using techniques comparable to previous publications. Therefore to address these issues HDAC and HAT activity, HDAC isoform expression and the substrates of HDAC and HAT containing complexes should be examined in samples obtained by bronchoscopy from smokers and non smokers with asthma characterised for corticosteroid responses.

7 Non-invasive assessment of inflammation in smokers with asthma

7.1 Introduction

7.1.1 Extended flow nitric oxide analysis

Non-invasive assessment of airway inflammation, through the measurement of exhaled nitric oxide at a flow rate of 50ml/sec, has developed into a useful exploratory endpoint in clinical trials since its discovery in the exhaled breath of subjects with asthma (47, 210). A raised exhaled nitric oxide concentration displays a degree of correlation with airway eosinophilia and therefore is thought to provide a quick method of assessment for this established indication for corticosteroid treatment (47). As a result it is likely that exhaled nitric oxide measurement will go on to become part of the routine assessment of subjects with asthma referred to secondary care.

Unfortunately current cigarette smoking markedly reduces exhaled nitric oxide levels at the standard flow rate of 50ml/sec (50) rendering the test less useful, both for the assessment of airway inflammation in smokers with asthma and as an exploratory endpoint in clinical trials. A recent publication suggests that a percentage change in exhaled nitric oxide correlates with asthma control and may therefore provide an alternative approach in smokers with asthma (221). However further work is required to corroborate this evidence and its place in the investigation of smokers with asthma.

An alternative approach involving examination of exhaled nitric oxide using multiple exhalation flow rates provides additional information beyond exhaled nitric oxide concentration. Mathematical modelling using the results from multiple flow rates, based on the assumption that the lungs can be divided into two compartments (composed of the conducting airways and the alveoli), has led to the development of a technique termed extended flow analysis. Extended flow analysis enables estimates to be derived for alveolar nitric oxide concentrations and flow independent measurements for the conducting airways

(airway wall nitric oxide flux (the rate of radial transport of nitric oxide across the airway into the exhaled air), airway wall concentration and diffusion rate) (222, 227, 255, 297). This approach has demonstrated elevated levels of alveolar nitric oxide in subjects with severe asthma (compared to subjects with mild asthma) (256, 257) and subjects with COPD (225, 258) (although this is not consistent (52)). Active smoking does not reduce alveolar nitric oxide levels in normal subjects (226, 259) and alveolar nitric oxide levels are equivalent in smokers and ex-smokers with COPD (52, 225). Therefore extended flow analysis may provide useful exploratory endpoints in the assessment of smokers with asthma.

However the calculation of alveolar nitric oxide and associated measurements is complicated by the existence of multiple methods of derivation (227). The original paper examining nitric oxide exchange mechanics used three high exhalation flow rates with plots of the elimination rate of nitric oxide against flow rate and subsequent linear regression through the plotted data (222). A similar method, performed using two low flow rates and prolonged exhalation allows for estimates to be derived for airway wall nitric oxide concentration, nitric oxide diffusion across the airway and airway nitric oxide flux levels (227, 255). An alternative method using non-linear regression enables the derivation of alveolar nitric oxide, airway wall nitric oxide concentration, nitric oxide diffusion and flux (255). Each of these models provides slightly different values for the derived parameters although normal values and values in subjects with asthma are available (table 7.1)(227). No research has been published to date examining extended flow nitric oxide analysis using these models in smokers with asthma.

Therefore to test the hypothesis that smokers with asthma have elevated levels of alveolar nitric oxide compared to non-smokers with asthma, reflecting their increased symptoms from asthma and alterations in other exhaled nitric oxide parameters (compared to non-smokers with asthma) the following study was undertaken. The use of an oral corticosteroid trial also allowed for the examination of the supplementary hypothesis that smokers with asthma display a restoration of alveolar nitric oxide levels towards the range present in non-smokers with asthma in response to corticosteroid therapy.

7.1.2 Exhaled breath condensate pH

Exhaled breath condensate (EBC) assessment, which involves the collection of expiratory gas vapours in a cooled tube, has been suggested to show promise as an exploratory endpoint in studies of asthma. Previous research has demonstrated that the pH of EBC is reduced during periods of exacerbation in asthma, returning to normal in parallel with clinical resolution (57). EBC pH also correlates negatively with induced sputum neutrophilia in subjects with COPD and sputum eosinophilia in non-smoking subjects with asthma (238). Smokers with asthma have previously been demonstrated to have lower EBC pH values than non smokers with asthma (58). EBC can be collected outside of the research laboratory for subsequent pH analysis and potentially represents a simple and useful non-invasive marker.

Therefore to examine the hypothesis that smokers with asthma display lower levels of exhaled breath condensate pH compared to non-smokers, and the additional hypothesis that following oral corticosteroids exhaled breath condensate pH is equivalent in smokers and non-smokers with asthma, the following study was undertaken.

7.2 Methods

7.2.1 Subjects

Subject characteristics, inclusion/exclusion criteria and recruitment methods were as described in the general methods chapter. All subjects provided informed consent and the study was approved by the West Glasgow Ethics Committee.

7.2.2 Study design

A full description of the study is provided in the general methods chapter. In brief, the subjects were recruited to a cross-sectional study with unblinded use of oral dexamethasone to determine corticosteroid sensitivity. Subjects performed extended flow nitric oxide analysis and EBC collection for pH

measurement before and at the completion of the corticosteroid trial. A small sub-set of patients attended one month after completion of the corticosteroid trial to investigate the duration of corticosteroid effects on extended flow nitric oxide parameters.

7.2.3 Measurements

A full description of the measurements is provided in the general methods chapter. Briefly, subjects were asked to refrain from eating and to avoid caffeine containing drinks within three hours of performing the tests. Smokers with asthma were also asked to refrain from smoking for three hours. Exhaled carbon monoxide measurements were performed to confirm abstinence from smoking. Inhaled medications were withheld consistent with available guidelines (246) to facilitate spirometry testing later in the study visit.

Nitric oxide measurements were performed at multiple flow rates (30, 50, 100, 150, 200, 250 & 300ml/sec) using a Niox-Flex analyser within built-in Flex-Flow programme and automatic flow regulator (Aerocrine AB, Sundbybergsvägen 9, SE-171 73 Solna, Sweden). The Niox-Flex meets joint ATS/ERS criteria for the measurement of on-line $F_{E_{NO}}$ (47) and the Flex-Flow programme automatically assesses the NO measurements against pre-set accuracy criteria. At flow rates above 30ml/sec the permitted deviation was +/- 10% and below 30ml/sec +/- 3ml/sec. Readings out with these boundaries were automatically rejected. The exhalation time for each flow rate was; 10 seconds for 30 ml/sec, 10 s for 50 ml/sec, 6 seconds for 100, 150, 200, 250 and 300 ml/sec. Three acceptable readings were obtained for each flow rate. A built-in delay prevented subjects performing the measurements at less than 30 second intervals. A system check was carried out after six attempts to ensure drift had not occurred in the measurement. Calibration was performed fortnightly using a certified nitric oxide gas cylinder. The results collected and displayed by the Flex-Flow programme were exhaled nitric oxide concentration ($F_{E_{NO}}$) and elimination rate of nitric oxide (V_{NO}). No reproducibility testing was performed.

Exhaled nitric oxide linear and non linear modelling was performed according to previously published methodology (222, 227, 255). This previous work utilises a 'two-compartment' model where the model divides the lung into a fixed area

airway and an expansible alveolar compartment combined with the assumption that NO is produced at a constant rate per unit volume in both compartments. This division is hoped to detected differences that are masked by the standard flow rate i.e. a subject with small airway inflammation may have an equivalent exhaled nitric oxide level to a subject with well controlled asthma (using the standard flow rate) but a significantly higher alveolar nitric oxide. The currently available models have been demonstrated to provide good estimates of alveolar nitric oxide, with levels in the range of that observed during endobronchial sampling and are also able to replicate the observed linear relationship of exhaled NO with flow rate. To calculate estimates for the desired parameters subjects exhale at several flow rates and the exhaled nitric oxide concentration, elimination rate of nitric oxide (amount of NO absorbed from the airway wall into the airstream) and exhalation flow rate are plotted followed by linear or non-linear fitting to the data (examples provided in figures 7.1 and 7.2).
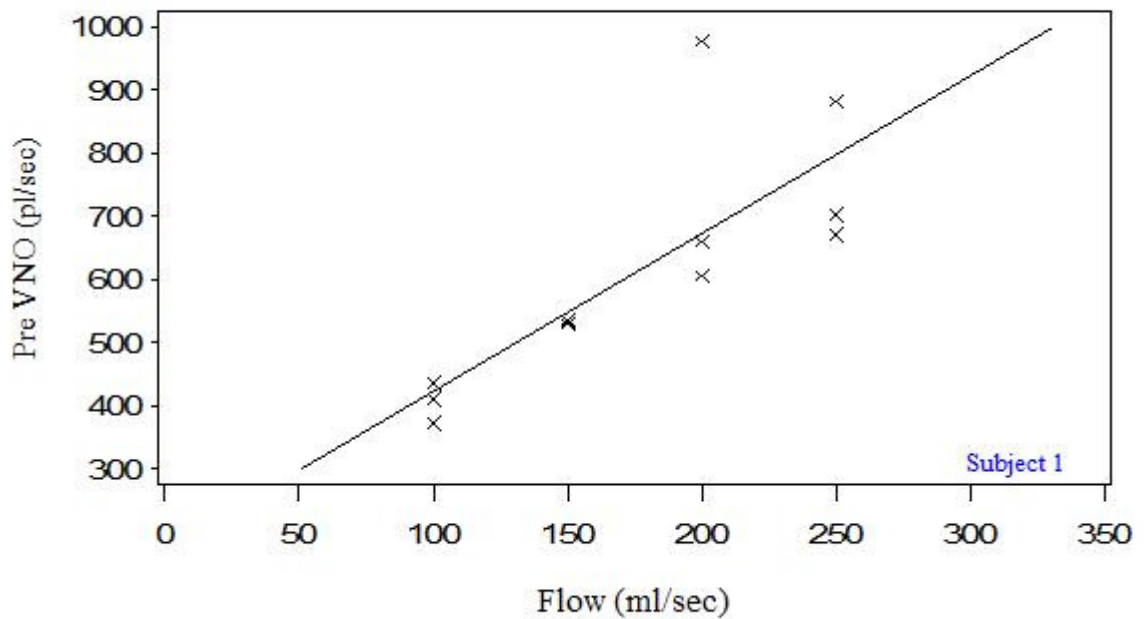
Figure 7.1 Plot of elimination rate of exhaled nitric oxide against exhalation flow rate.

Line represents linear regression through the data points. The slope of the line reflects the alveolar nitric oxide concentration and the y-intercept the airway wall flux.

V_{NO}; elimination rate of exhaled nitric oxide, pl/sec; picolitres per second, ml/sec; millilitres per second
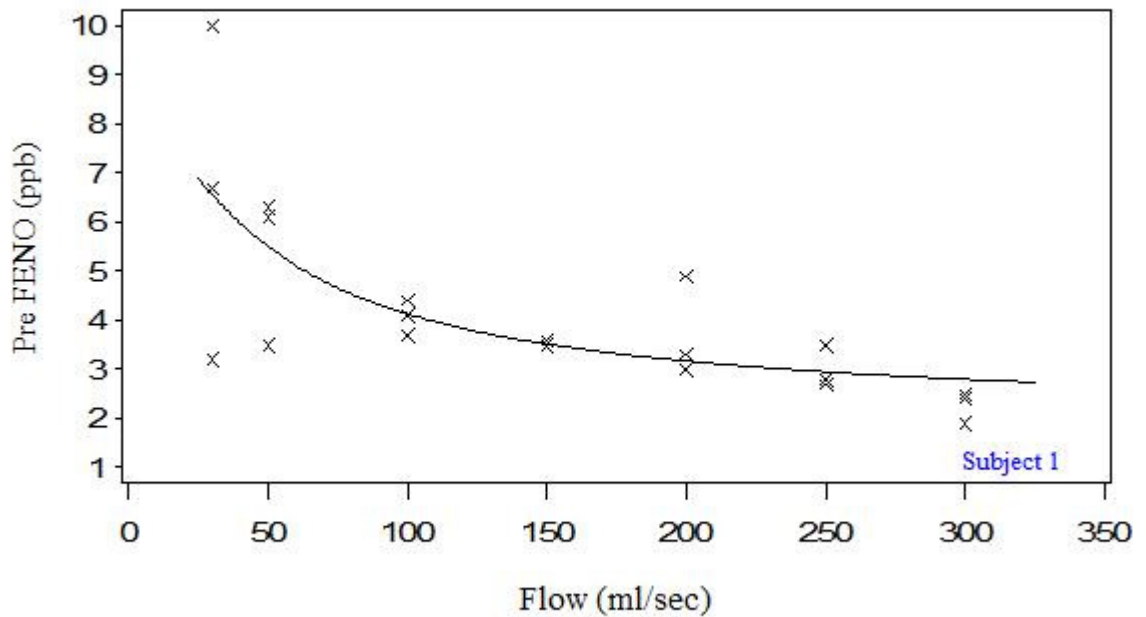
Figure 7.2 Plot of exhaled nitric oxide concentration against exhalation flow rate. Line represents non-linear regression through the data points.

FE$_{NO}$; exhaled nitric oxide concentration, ppb; parts per billion, ml/sec; millilitres per second

Linear modelling was performed initially according to (222) using V$_{NO}$ results from 100, 200 and 300 ml/sec flow rates with subsequent comparison against results derived using data from 100, 150, 200 & 250 ml/sec and 100, 150, 200, 250 & 300 ml/sec flow rates. Plotting V$_{NO}$ results against exhalation flow rates and linear regression allows derivation of alveolar nitric oxide and airway flux according to the following equation (see figure 7.1):

$$V_{NO} = Calv.V_E + J'aw_{NO}$$

Where V$_{NO}$= elimination rate of exhaled nitric oxide (ml/s), V$_E$ = exhalation flow rate (ml/s), Calv= alveolar NO concentration (ppb), J'aw$_{NO}$=maximum NO flux (pl/sec).

Where alveolar nitric oxide corresponded to the gradient of the line and flux was obtained from the y-intercept of the line. If a subject's data provided a negative value for alveolar nitric oxide following regression their data was not included in the final analysis as this was felt to represent a test error.

Airway wall nitric oxide diffusion and concentration was obtained by linear regression using the V_{NO} and F_{ENO} results from the 30 and 50ml/sec flow rates. V_{NO} was plotted against F_{ENO} followed by linear regression. Nitric oxide flux was obtained from the y-intercept and nitric oxide diffusion from the reciprocal of the gradient of the slope. Airway wall concentration was obtained from the relationship (255):

$$J'aw_{NO} = Caw_{NO}Daw_{NO}$$

Where J'aw$_{NO}$=maximum NO flux (pl/sec), Caw$_{NO}$=airway wall concentration (ppb), Daw$_{NO}$=diffusion from airway wall to airway (pl/sec/ppb)

Nonlinear regression was performed using the F_{ENO} data from all flow rates with the restriction of positive boundaries for all parameters. Plotting F_{ENO} against V_E (see figure 7.2) and solving for the following equation enabled the derivation of estimates for alveolar nitric oxide, airway nitric oxide concentration, airway wall nitric oxide diffusion and flux parameters. (255):

$$F_{ENO} = Caw_{NO} + (Calv - Caw_{NO})e^{(-DawNO/V_E)}$$

Where F$_{ENO}$=exhaled NO concentration (ppb), Caw$_{NO}$=airway wall concentration (ppb), Daw$_{NO}$=diffusion from airway wall to airway (pl/sec/ppb), Calv =alveolar NO level (ppb), V$_E$=flow rate of exhalation (ml/sec)

An estimate of airway flux was subsequently derived for this model from the relationship (227):

$$J'aw_{NO} = Caw_{NO}Daw_{NO}$$

Previously published results using the presented models are presented in table 7.1. This data will provide reference ranges for the data presented in the results section.

	Normal adults	Non-smokers with asthma (No ICS)	Non-smokers with asthma (ICS)
Linear modelling			
C_{alv} (ppb)	1.0-1.9	1.1-1.5	1.2-1.9
J_{aw} (pl/s)	600-1200	2500-6512	700-2416
C_{aw} (ppb)	75-98	255	108-144
D_{aw} (pl/s/ppb)	7.7-11.0	25.5	11.8-22.3
Non-linear modelling			
C_{alv} (ppb)	3.2-5.0		
J_{aw} (pl/s)	1020	6512	2416
C_{aw} (ppb)	149	255.3	108.3
D_{aw} (pl/s/ppb)	5.7-7.4	25.5	22.3

Table 7.1 Ranges for normal adults, non-smoking subjects with asthma not treated and treated with inhaled corticosteroid.

Based on previous published results (52, 227, 255, 257, 298, 299). Mean values for groups presented. ICS; inhaled corticosteroid treated, Calv; alveolar nitric oxide, Jaw; airway nitric oxide flux, Caw; nitric oxide concentration in airway wall, Daw; airway wall diffusion rate of nitric oxide, ppb; parts per billion, pl/s; picolitres per second, pl/s/ppb; picolitres per second per parts per billion.

Exhaled breath condensate was collected using a Jaeger EcoScreen®. The subjects performed tidal breathing for ten minutes whilst wearing a nose clip. De-aeration of the sample was performed for ten minutes using argon as per recommended guidelines and previous published methodology (56, 57). Sample pH was measured immediately on completion of de-aeration following a twenty second period to allow stabilisation of the probe reading in the sample. No reproducibility testing was performed.

7.2.4 Statistical analysis

Parametric data was assessed using t testing and non parametric using Mann-Whitney testing. All extended flow analysis are presented as median (IQR) and all comparisons are between smokers and non smokers. Ex-smokers were not included in formal comparison analyses due to the small number of subjects in

this group. All data was treated as exploratory. α was set at 0.05. Analysis and linear and non-linear modelling was performed using SAS v 9.1 (TS1M3) for Windows (SAS Institute Inc., NC, USA). Results are presented as median (interquartile range) unless stated otherwise.

7.3 Results

7.3.1 Baseline comparisons

The baseline characteristics are discussed in detail in chapter 5 sections 5.3.1 to 5.3.4. A brief review of the relevant findings will be presented here.

The subjects were well matched at baseline but mean ACQ score and inhaled corticosteroid dose were higher in smokers with asthma. Smokers with asthma had a smaller bronchodilator response compared to non-smokers with asthma. 22 smokers, 10 ex-smokers and 21 non-smokers were recruited to the study. 20 smokers, 9 ex-smokers and 17 non-smokers completed the oral corticosteroid trial. Non-smokers with asthma made a significant lung function response to oral corticosteroids in contrast to smokers and ex-smokers with asthma.

19 smokers, 9 ex-smokers and 20 non-smokers with asthma were able to perform acceptable nitric oxide measurements at baseline and 19 smokers, 7 ex-smokers and 17 non-smokers with asthma performed acceptable measurements post corticosteroid trial. 16 smokers, 9 ex-smokers and 15 non-smokers with asthma performed acceptable exhaled nitric oxide measurements after a period of one month had elapsed from the oral corticosteroid trial.

7.3.2 Exhaled nitric oxide-FE_{NO50}

Clear differences were evident between smokers and non-smokers with asthma at baseline (table 7.2 and figure 7.3), on completion of the corticosteroid trial and one month after oral corticosteroids. There appeared to be a small reduction in FE_{NO50} in smokers with asthma in response to oral corticosteroids.

	Smokers	Ex-smokers	Non-smokers
FE_{NO50} pre steroid (ppb)	11.1 ‡ (3.6, 13.5)	19.8 (15.8, 43.5)	32.8 (17.7, 73.2)
FE_{NO50} post steroid (ppb)	6.1 † (3.3, 8.1)	11.4 (7.0, 22.0)	12.4 (10.1, 22.0)
FE_{NO50} 1 month post steroid (ppb)	8.4 * (4.6, 13.9)	20.6 (8.9, 23.5)	13.5 (6.5, 23.7)

Table 7.2 Exhaled nitric oxide measured at standard flow rate of 50ml/sec.

FE_{NO50}; exhaled nitric oxide concentration at 50ml/sec, ppb; parts per billion. Data presented as median (IQR). p values refer to comparison of smokers and non smokers. *; $p<0.05$, †; $p<0.01$, ‡, $p<0.001$.
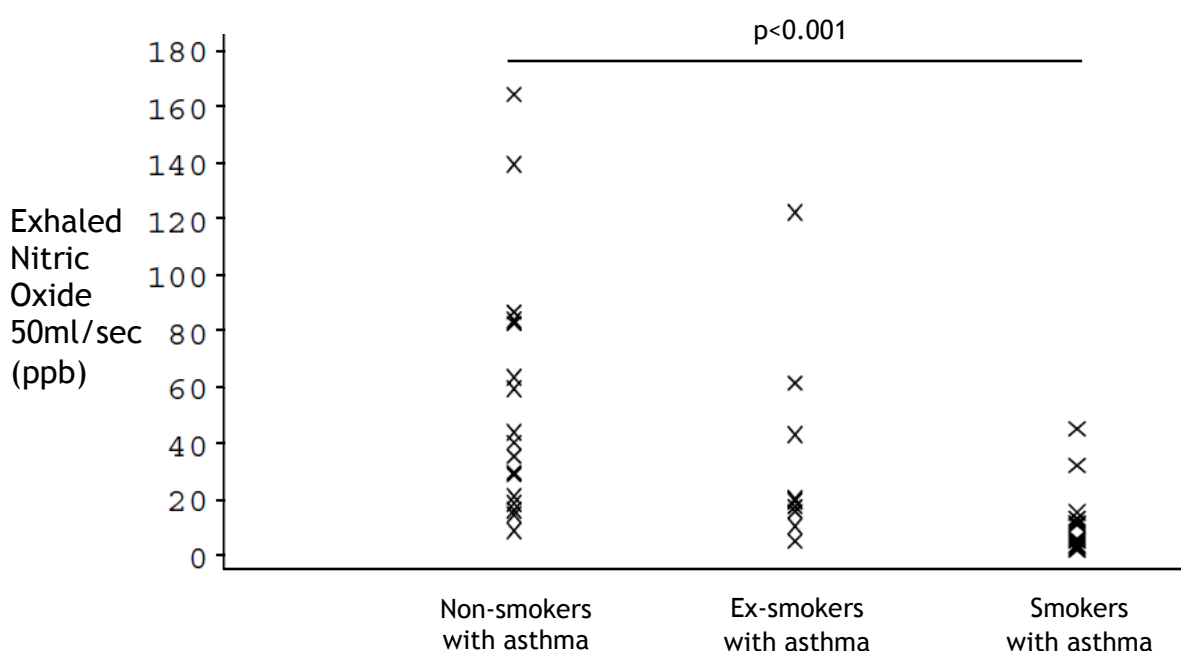
Figure 7.3 Baseline exhaled nitric oxide (FE_{NO50}) levels.

ppb; parts per billion.

7.3.3 Exhaled breath condensate pH

No significant differences in EBC pH were evident between smokers and non smokers with asthma at baseline, post oral corticosteroid trial or one month later (table 7.3). When within group responses to oral corticosteroid trial were compared there was no significant difference (smokers; median change 0.05 (IQR -0.08, 0.17) non-smokers; 0.03 (-0.09, 0.15), p=0.770). EBC pH one month post corticosteroid was equivalent to EBC pH at baseline and post corticosteroid trial.

	Smokers	Ex smokers	Non-smokers
EBC pH Pre steroid	7.22 (7.16, 7.27)	7.29 (7.24, 7.33)	7.26 (7.16, 7.36)
EBC pH Post steroid	7.29 (7.13, 7.36)	7.36 (7.20, 7.38)	7.34 (7.21, 7.36)
EBC pH 1 month post steroid	7.21 (7.15, 7.27)	7.25 (7.25, 7.30)	7.25 (7.18, 7.31)

Table 7.3 EBC pH at baseline, post corticosteroid trial and one month after oral corticosteroid trial.

Data presented as median (IQR). EBC; exhaled breath condensate.

7.3.4 Extended flow nitric oxide

7.3.4.1 Alveolar Nitric Oxide and Airway Wall Flux

7.3.4.1.1   Linear analysis

Smokers with asthma displayed a lower median level of alveolar nitric oxide compared to non-smokers with asthma at baseline although this difference was lost after the oral corticosteroid trial (table 7.5). Smokers with asthma also had significantly lower levels for median nitric oxide flux pre and post steroid. The median alveolar nitric oxide concentration and flux in ex-smokers with asthma was intermediate to the non-smokers and smokers with asthma at baseline. However alveolar nitric oxide post corticosteroid appeared to be lower than that observed in smokers and the level of nitric oxide flux was equivalent to that observed in non-smokers with asthma.

	Smokers	Ex smokers	Non-smokers
C_{alv} pre steroid (ppb)	1.42 * (0.43, 2.01)	1.71 (0.59, 2.79)	2.45 (1.11, 3.52)
J_{aw} pre steroid (pl/s)	572.8 † (216.7, 734.4)	987.6 (695.2, 3308.3)	1535.0 (784.6, 3495.6)
C_{alv} post steroid (ppb)	1.79 (0.68, 2.38)	0.58 (0.40, 0.76)	1.89 (1.37, 2.93)
J_{aw} post steroid (pl/s)	147.6 † (65.0, 458.0)	570.0 (413.9, 932.6)	577.0 (456.9, 1361.7)

Table 7.5 Linear regression analysis results for 100, 200, 300 ml/sec.

C_{alv}; alveolar nitric oxide, J_{aw}; nitric oxide flux. ppb; parts per billion, pl/s; picolitres per second. p values refer to comparison of smokers and non smokers. *; $p < 0.05$, †; $p < 0.01$, ‡, $p < 0.001$.

Performance of linear regression with data from multiple additional flows (100, 150, 200 & 250 and subsequently with the addition of data from 300 ml/sec) altered the concentrations derived for alveolar nitric oxide. For example, median alveolar nitric oxide values for smokers with asthma at baseline derived using four flows was 1.53 ppb (IQR 0.09, 2.50) and five flows was 1.03 ppb (-0.03, 2.02). However the use of data from extra flow rates did not remove the significant difference between the groups at baseline or affect the narrowing of the difference following the oral corticosteroid trial. Values derived for nitric oxide flux were not obviously affected by the use of data from additional flow rates.

Linear regression using the low flow rates of 30 and 50ml/sec produced different values for nitric oxide flux compared to higher flows. However in keeping with the previous models, smokers with asthma had significantly lower nitric oxide flux levels at baseline (smokers 396.8 pl/s (IQR 68.8, 834.7), non-smokers 1984.6 pl/s (1257.9, 5580.5), p<0.001) and post oral corticosteroid (smokers 207.4 pl/s (-8.4, 635.2), non-smokers 587.2 pl/s (383.2, 1342.4), p=0.004) using this approach. When response to oral corticosteroids was compared smokers with asthma made a significantly smaller reduction in nitric oxide flux (smokers change -77.2 pl/s (-284.3, 139.2), non-smokers change -1363.8 pl/s (-4094.8, -678.0), p=0.003). Ex-smokers with asthma displayed airway wall concentrations and flux levels similar to those observed in non-smokers with asthma.

7.3.4.1.2   Non-linear regression analysis

Non-linear regression produced equivalent median alveolar nitric oxide concentrations in smokers and non-smokers with asthma at baseline (smokers 1.39 ppb (IQR 0.00, 1.95), non-smokers 0.78 ppb (0.00, 1.69), p=0.760) and post oral corticosteroid trial (smokers 0.97 ppb (0.00, 2.04), non-smokers 1.25 ppb (0.43, 2.14), p=0.360) (table 7.6 & figure 7.4). No significant difference was evident between smokers and non-smokers with asthma when change in alveolar nitric oxide in response to oral corticosteroid was compared (smokers median change 0.00 ppb (IQR -0.61, 0.64), non-smokers median change 0.43 ppb (0.00, 1.27), p=0.240). Ex-smokers with asthma had alveolar nitric oxide levels slightly lower than those observed in smokers with asthma.
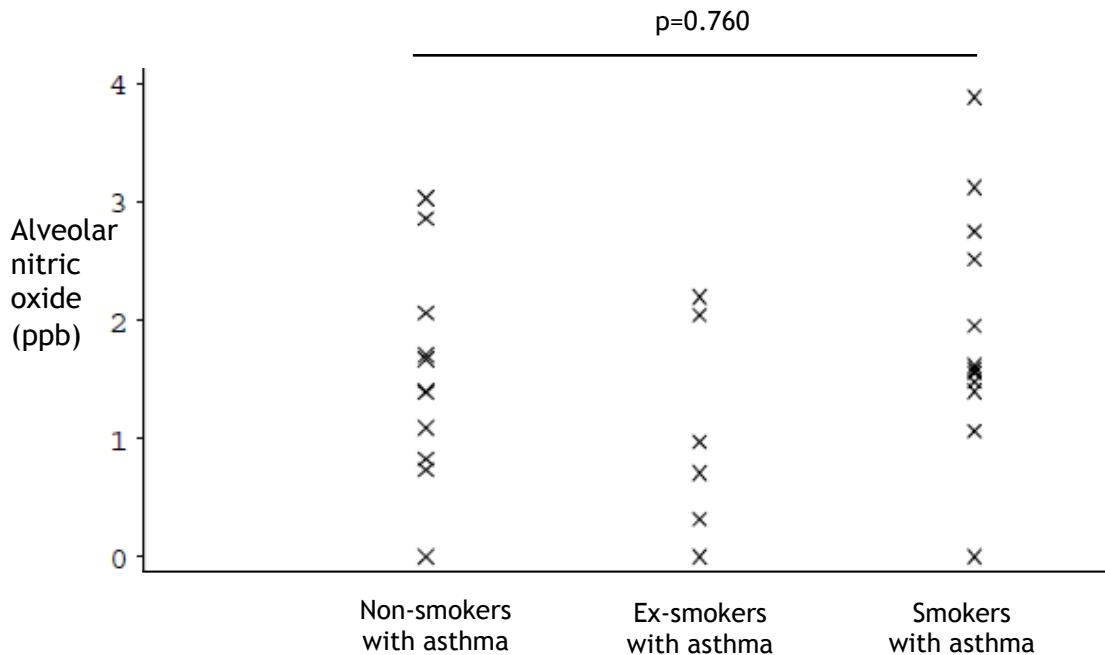
Figure 7.4 Baseline alveolar nitric oxide (C$_{alv}$)-non-linear modelling.

ppb; parts per billion.

	Smokers	**Ex smokers**	**Non-smokers**
C$_{alv}$ pre steroid (ppb)	1.39 (0.00, 1.95)	0.32 (0.00, 0.97)	0.78 (0.00, 1.69)
J$_{aw}$ pre steroid (pl/s)	697.4 [†] (322.5, 1204.8)	1278.3 (715.6, 4221.8)	2087.8 (1093.4, 5033.6)
C$_{alv}$ post steroid (ppb)	0.97 (0.00, 2.04)	0.61 (0.00, 0.78)	1.25 (0.43, 2.14)
J$_{aw}$ post steroid (pl/s)	335.7 [†] (278.7, 732.0)	947.4 (438.3, 1302.5)	676.9 (608.2, 1132.4)

Table 7.6 Non linear regression analysis.

Results presented as median (IQR). C$_{alv}$; alveolar nitric oxide, J$_{aw}$; nitric oxide flux. ppb; parts per billion, pl/s; picolitres per second. p values refer to comparison of smokers and non smokers. *; p<0.05, †; p<0.01, ‡, p<0.001.

Nitric oxide flux values were significantly lower in smokers with asthma at baseline (smokers 697.4 pl/s (IQR 322.5, 1204.8), non-smokers 2087.8 pl/s (1093.4, 5033.6), p=0.002) and post oral corticosteroids (smokers 335.7 pl/s (278.7, 732.0), non-smokers 676.9 pl/s (608.2, 1132.4), p=0.004) (table 7.6 and figure 7.5). Smokers with asthma also demonstrated a trend to a reduction in nitric oxide flux in response to oral corticosteroids (smokers within group median change -260.1 pl/s (IQR -492.1, 33.1), p=0.064). This was in contrast to non-

smokers where a large and strongly significant reduction was observed (non-smokers -1363.0 pl/s (-3831.0, -725.0), p<0.001). Baseline levels for nitric oxide flux in ex-smokers with asthma were higher than those observed in smokers with asthma and suggested a trend towards the levels observed in non-smokers with asthma.
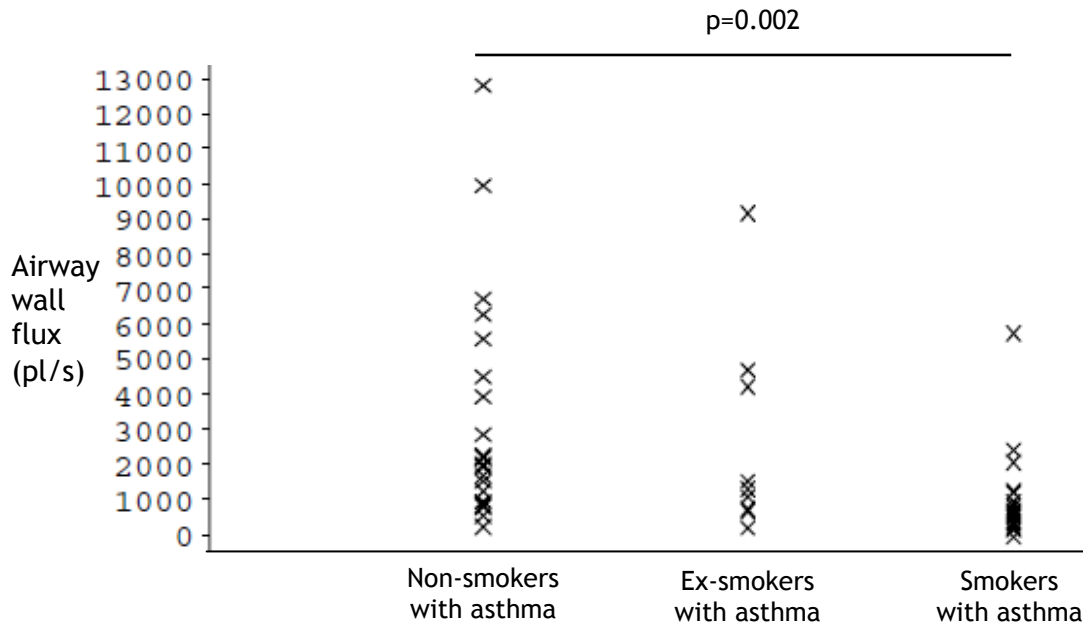
Figure 7.5 Baseline nitric oxide flux (J$_{aw}$)-non-linear modelling.

pl/s; picolitres per second.

7.3.4.2 Airway wall Nitric Oxide concentration and Nitric Oxide diffusion

7.3.4.2.1   Linear regression

The airway wall nitric oxide concentration was significantly lower in smokers with asthma at baseline (smokers 8.6 ppb (IQR -4.4, 53.3), non-smokers 147.6 ppb (59.7, 243.3), p=0.002) (table 7.7). However post oral corticosteroids this difference was narrowed and became non-significant, although a trend to a difference was evident (smokers 15.0 ppb (0.2, 37.0), non-smokers 45.0 ppb (20.8, 89.4), p=0.08). Smokers with asthma made a significantly smaller reduction in airway wall nitric oxide concentration in response to oral corticosteroids when assessed by linear modelling (smokers median change -1.47 ppb (IQR -24.4, 19.9), non-smokers -111.6 ppb (-188.3, 2.4), p=0.030). Levels obtained for ex-smokers at baseline were higher than smokers and were similar to the levels observed in non-smokers (ex-smokers; baseline 166.9 ppb (43.6, 253.1), post corticosteroid 86.0 ppb (57.4, 118.9)).

Smokers with asthma had a trend to lower rates of airway wall nitric oxide diffusion compared to non-smokers at baseline (smokers 11.7 pl/s/ppb (IQR - 19.2, 17.7), non-smokers 18.0 pl/s/ppb (7.3, 26.0), p=0.070), but no difference was evident post oral corticosteroid trial (smokers 0.8 pl/s/ppb (-43.3, 30.4), non-smokers 12.2 pl/s/ppb (4.5, 20.0), p=0.240) (table 7.7). No significant difference in airway wall nitric oxide diffusion response to oral corticosteroids was evident when the two groups were compared. Ex-smokers diffusion levels were equivalent to smokers and did not appear to significantly change in response to oral corticosteroids.

	Smokers	Ex smokers	Non-smokers
C_{aw} **pre steroid** (ppb)	8.6 [†] (-4.4, 53.3)	166.9 (43.6, 253.1)	147.6 (59.7, 243.3)
D_{aw} **pre steroid** (pl/s/ppb)	11.7 (-19.2, 17.7)	6.0 (0.8, 16.6)	18.0 (7.3, 26.0)
C_{aw} **post steroid** (ppb)	15.0 (0.2, 37.0)	86.0 (57.4, 118.9)	45.0 (20.8, 89.4)
D_{aw} **post steroid** (pl/s/ppb)	0.8 (-43.3, 30.4)	6.7 (3.1, 11.3)	12.2 (4.5, 20.0)

Table 7.7 Airway wall concentration and diffusion of nitric oxide produced by linear regression using 30 and 50ml/sec flow rates.

C_{aw}; airway wall concentration of nitric oxide, D_{aw}; airway wall nitric oxide diffusion, ppb; parts per billion, pl/s/ppb; picolitres per second per parts per billion. p values refer to comparison of smokers and non smokers. †; p<0.01.

7.3.4.2.2   Non-linear regression

Smokers with asthma had significantly lower airway wall nitric oxide concentrations at both baseline (smokers 25.9 ppb (IQR 7.1, 32.2), non-smokers 117.8 ppb (62.0, 173.5), p<0.001) (table 7.8) and post oral corticosteroid trial (smokers 10.8 ppb (7.0, 25.5), non-smokers 38.7 ppb (27.2, 81.6), p=0.021). When responses to the corticosteroid trial were compared it was evident that non-smokers with asthma made a significantly greater reduction in airway wall nitric oxide concentration (smokers median change -5.5 ppb (IQR -18.9, 0.9), non-smokers -54.1 ppb (-116.2, -24.8), p=0.020). Ex-smokers demonstrated a trend towards the levels observed in non-smokers with asthma at baseline. No change in airway wall nitric oxide concentrations were observed in response to oral corticosteroids in the ex-smokers with asthma.

Nitric oxide diffusion was equivalent in both groups at baseline and post oral corticosteroid trial. The changes in nitric oxide diffusion in response to the corticosteroid trial were equivalent and small in nature (smokers median change -8.9 pl/s/ppb (-28.0, 43.3), non-smokers -6.3 pl/s/ppb (-21.1, 0.0), p=0.700). Ex-smokers demonstrated parity for nitric oxide diffusion at baseline with an apparent reduction in response to oral corticosteroids.

	Smokers	**Ex smokers**	**Non-smokers**
C_{aw} **pre steroid** (ppb)	25.9 [‡] (7.1, 32.2)	62.9 (35.9, 269.3)	111.8 (62.0, 173.5)
D_{aw} **pre steroid** (pl/s/ppb)	25.7 (6.0, 46.5)	24.8 (1.0, 33.4)	27.8 (15.7, 36.5)
C_{aw} **post steroid** (ppb)	10.8 [*] (7.0, 25.5)	64.3 (17.0, 177.7)	38.7 (27.2, 81.6)
D_{aw} **post steroid** (pl/s/ppb)	29.8 (1.0, 55.4)	6.3 (1.0, 20.1)	16.2 (7.5, 28.9)

Table 7.8 Airway wall concentration and diffusion of nitric oxide produced by non-linear regression.

C_{aw}**; airway wall concentration of nitric oxide, D_{aw}; airway wall nitric oxide diffusion, ppb; parts per billion, pl/s/ppb; picolitres per second per parts per billion. p values refer to comparison of smokers and non smokers. *; $p<0.05$, ‡; $p<0.001$.**

7.3.4.3 Comparison of linear and non-linear models

Comparison between the linear and non-linear methods was possible for the baseline results. A Bland-Altman plot for alveolar nitric oxide (figure 7.6), reveals that the models provided very different results for alveolar nitric oxide levels with disagreements of up to 8 ppb and a more routine difference of about 4 ppb present.
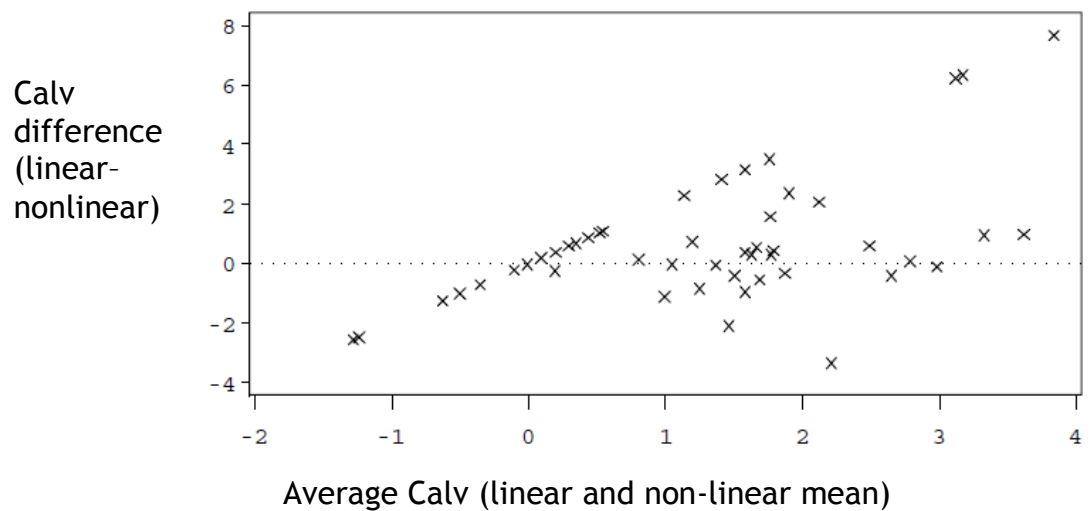
Figure 7.6 Bland-Altman plot of Alveolar nitric oxide (Calv) difference (linear-non-linear) against alveolar nitric oxide (Calv) average for linear and non-linear models

7.3.4.4 Impact of corticosteroids on extended flow measurements at one month

Performance of extended flow nitric oxide measurements one month post oral corticosteroid trial allowed a simple examination of the duration of effect of the corticosteroid trial on these markers of inflammation.

When alveolar nitric oxide derived by linear modelling (using three flow rates) was examined it was evident that corticosteroids continued to have an effect at one month. Alveolar nitric oxide was equivalent in smokers and non-smokers with asthma in contrast to baseline (table 7.9). Comparing the change in alveolar nitric oxide from pre corticosteroid visit to one month post corticosteroids demonstrated that alveolar nitric oxide significantly increased in the smokers with asthma (smokers median change 1.34 ppb (IQR 0.21, 2.31), non-smokers -0.52 ppb (-1.21, 0.18), p=0.007). Airway nitric oxide flux at one month post oral corticosteroid trial continued to be significantly lower in smokers with asthma (table 7.9). However nitric oxide flux showed a reduction in both groups with a trend to a larger reduction in non-smokers with asthma (smokers median change -136.9 pl/s (IQR -429.7, 209.6), non-smokers change -281.7 pl/s (-875.0, -18.7), p=0.060). Ex-smokers with asthma demonstrated continued suppression of their alveolar nitric oxide level at one month whilst their flux had recovered towards baseline levels and showed parity with non-smokers with asthma.

	Smokers	Ex smokers	Non-smokers
C_{alv} **pre steroid** (ppb)	0.69 [*] (0.19, 1.93)	0.87 (0.07, 2.56)	2.19 (1.10, 3.34)
J_{aw} **pre steroid** (pl/s)	572.8 [‡] (216.7, 734.4)	987.6 (695.2, 3047.5)	1563.7 (797.4, 3659.0)
C_{alv} **1 month post steroid** (ppb)	1.82 (1.08, 2.65)	0.00 (-0.63, 0.69)	1.37 (0.13, 3.00)
J_{aw} **1 month post steroid** (pl/s)	462.2 [†] (215.1, 671.7)	1129.6 (433.2, 1235.7)	1213.7 (591.3, 2637.4)

Table 7.9 Variation in alveolar nitric oxide and airway nitric oxide for linear modelling using 100, 200 & 300ml/sec flow rates.

Results presented as median (IQR). C_{alv}; alveolar nitric oxide, J_{aw}; nitric oxide flux. ppb; parts per billion, pl/s; picolitres per second. [*]; $p<0.05$, [†]; $p<0.01$, [‡]; $p<0.001$.

Alveolar nitric oxide estimates generated by non-linear modelling demonstrated a trend to higher alveolar nitric oxide levels at one month in the smokers with asthma (smokers 1.61 ppb (IQR 0.0, 2.5), non-smokers 0.23 ppb (0.0, 0.6), p=0.059) (table 7.10). When alveolar nitric oxide change from baseline to one month post corticosteroid was compared there appeared to be a slight rise in the smokers with asthma (smokers median change 0.63 ppb (IQR -0.01, 1.20), non-smokers 0.00 ppb (-1.67, 0.23), p=0.060).

Non-linear modelling revealed that smokers with asthma continued to have significantly lower nitric oxide flux levels after one month (smokers 378.7 pl/s (IQR 130.2, 902.0), non-smokers 1379.8 pl/s (591.9, 3324.2), p=0.006) (table 7.10). No difference was evident when change in flux from baseline to one month post oral corticosteroid trial was compared (smokers median change -92.3 pl/s (IQR -607.7, 78.9), non-smokers -358.7 pl/s (-1161.9, 33.5), p=0.380). Reflecting the linear modelling result, non-linear modelling also suggested that ex-smokers with asthma displayed suppressed levels of alveolar nitric oxide at one month. Nitric oxide flux levels had also returned to the range observed at baseline in ex-smokers with asthma at one month.

	Smokers	Ex smokers	Non-smokers
C_{alv} **pre steroid** (ppb)	1.39 (0.00, 1.95)	0.32 (0.00, 0.97)	0.78 (0.00, 1.69)
J_{aw} **pre steroid** (pl/s)	697.4 [†] (322.5, 1204.8)	1278.3 (715.6, 4221.8)	2087.8 (1093.4, 5033.6)
C_{aw} **pre steroid** (ppb)	25.9 [‡] (7.1, 32.2)	62.9 (35.9, 269.3)	111.8 (62.0, 173.5)
D_{aw} **pre steroid** (pl/s/ppb)	25.7 (6.0, 46.5)	24.8 (1.0, 33.4)	27.8 (15.7, 36.5)
C_{alv} **1 month post steroid** (ppb)	1.61 (0.00, 2.51)	0.00 (0.00, 0.18)	0.23 (0.00, 0.58)
J_{aw} **1 month post steroid** (pl/s)	378.7 [†] (130.2, 902.0)	1290.8 (280.1, 1611.3)	1379.8 (591.9, 3324.2)
C_{aw} **1 month post steroid** (ppb)	39.5 (8.5, 113.9)	91.8 (44.5, 194.3)	60.6 (36.8, 152.2)
D_{aw} **1 month post steroid** (pl/s/ppb)	1.5 (1.0, 25.4)	5.8 (1.0, 22.4)	14.9 (1.0, 29.8)

Table 7.10 Variation in alveolar nitric oxide, airway nitric oxide flux, concentration and diffusion for non-linear modelling.

Results presented as median (IQR). C_{alv}; alveolar nitric oxide, J_{aw}; nitric oxide flux, C_{aw}; airway wall concentration of nitric oxide, D_{aw}; airway wall nitric oxide diffusion. ppb; parts per billion, pl/s; picolitres per second pl/s/ppb; picolitres per second per parts per billion. †; $p<0.01$, ‡, $p<0.001$.

In contrast to baseline, airway wall nitric oxide (derived by non-linear modelling) was equivalent in smokers and non-smokers with asthma at one month. This narrowing of the difference appeared to be partly due to an increase in this marker in smokers with asthma and a sustained suppression in non-smokers (one month post levels; smokers 39.5 ppb (8.5, 113.9), non-smokers 60.6 ppb (36.8, 152.2), p=0.370) (table 7.10). No obvious difference was evident in nitric oxide diffusion one month post oral corticosteroid trial when smokers and non-smokers were compared. However levels appeared to be lower in smokers with asthma compared to baseline (smokers 1.5 pl/s/ppb (1.0, 25.4), non-smokers 14.9 pl/s/ppb (1.0, 29.8), p=0.110). Airway wall nitric oxide concentrations in ex-smokers with asthma appeared to be higher than non-smokers with asthma at one month post oral corticosteroid trial.

Airway wall concentrations at one month, derived using low flows and linear modelling, were equivalent (smokers 16.7 ppb (-4.7, 109.6), non-smokers 69.7 ppb (24.2, 145.7), p=0.170). Comparison of the change in airway wall

concentrations demonstrated a trend to a larger reduction in non-smokers with asthma (smokers -5.1 ppb (-19.9, 64.1), non-smokers -37.9 ppb (-195.5, 61.8), p=0.055). Nitric oxide diffusion was significantly lower in smokers with asthma (smokers 2.6 pl/s/ppb (-15.4, 9.2), non-smokers 12.9 pl/s/ppb (4.6, 20.5), p=0.026). However when median change from baseline was examined no difference in response was evident (smokers 7.3 pl/s/ppb (IQR -11.4, 17.9), non-smokers -1.6 pl/s/ppb (-13.3, 10.4), p=0.400). In contrast to the result derived by non-linear modelling, ex-smokers with asthma airway wall concentration at one month post oral corticosteroid was close to the level observed in smokers with asthma (ex-smokers one month post corticosteroid 15.9 ppb (-19.3, 112.0)).

7.4 Discussion

Smokers with asthma display a reduced response to inhaled and oral corticosteroids (5-10, 22-24). This altered response is associated with worse asthma control reflected in the higher ACQ scores of smokers with asthma (12). New treatments are currently being developed for corticosteroid resistant airway obstruction and may be useful in the management of this group. However to justify the cost and effort involved in performing definitive trials for these medications supportive evidence will initially be required from small exploratory trials in smokers with asthma. Therefore an exploratory endpoint that detects subtle anti-inflammatory effects which do not rapidly translate into lung function changes in smokers with asthma would be a useful additional test for the standard short exploratory trial. I chose to examine both exhaled breath condensate pH and extended flow nitric oxide analysis based on the hypothesis that both could provide a non-invasive test that reflects the poorly controlled inflammation present in smokers with asthma.

Extended flow analysis provides additional insights into airway nitric oxide metabolism, compared to the standard measurement of exhaled nitric oxide at a flow rate of 50ml/sec. Active smoking does not appear to reduce the alveolar nitric oxide concentration (226, 259) and elevated alveolar nitric oxide has been detected in subjects with COPD (225, 258). Based on this work and the potential ability of extended flow analysis to provide further additional insights in smokers with asthma and that it may correlate with the increased symptoms present in

smokers with asthma I felt that it was worthy of study. The data produced by linear modelling demonstrates that smokers with asthma have reduced alveolar nitric oxide, nitric oxide flux and airway wall diffusion levels compared to non-smokers with asthma and, when compared to previous research, perhaps lower alveolar nitric oxide levels than healthy non-smokers (table 7.1). The difference in alveolar nitric oxide levels in smokers and non-smokers with asthma is novel and contrasts with previous research in normal smokers (226) and COPD (52, 225). The oral corticosteroid trial demonstrates that the difference in alveolar nitric oxide levels between smokers and non-smokers with asthma (as determined by linear modelling) can be reduced by oral corticosteroids and this effect persists for at least one month.

In contrast, non-linear modelling demonstrates equivalency for alveolar nitric oxide in smokers and non-smokers with asthma. This result is consistent with previous work in normal smokers (226) and smokers and ex-smokers with COPD (52). Again alveolar nitric oxide levels were not raised in reflection of the increased symptoms in smokers with asthma and no clear change in alveolar nitric oxide was evident in either group in response to high dose corticosteroids leading one to question the usefulness of this endpoint. When the results from the linear and non-linear models were compared by Bland-Altman plot it was evident that the models could not be regarded as interchangeable.

Airway nitric oxide flux derived by both linear and non-linear modelling demonstrates a clear difference for this endpoint between smokers and non-smokers with asthma. Smokers with asthma display nitric oxide flux levels close to those previously observed in normal non-smokers. Nitric oxide flux also demonstrates sensitivity to oral corticosteroids in both smokers and non-smokers with asthma. The evidence of clear change in smokers with asthma nitric oxide flux following oral corticosteroids when derived by linear modelling and a trend to a reduction when derived by non-linear modelling is intriguing and suggests that nitric oxide flux is worthy of further study. Non-linear and linear modelling also revealed the novel finding that airway wall nitric oxide concentrations are significantly lower in smokers with asthma at baseline, post oral corticosteroid trial and at one month post oral corticosteroid in contrast to linear modelling. Airway wall nitric oxide, derived by both linear and non-linear modelling, appears to be sensitive to oral corticosteroids with non-smokers making

significantly greater reductions in response to oral corticosteroids. Smoking did not affect airway wall diffusion for nitric oxide and this is consistent with previously published work in normal smokers (226, 259) and ex-smoking subjects with COPD (52, 225).

The finding of reduced airway nitric oxide flux, nitric oxide airway wall concentrations and alveolar nitric oxide (when derived by linear modelling) in smokers with asthma is intriguing and it is tempting to speculate on possible causes. This study is not able to demonstrate the mechanism(s) by which this occurs but possible causes include cigarette smoke induced consumption of nitric oxide (51, 52), competition for required substrates by other inflammatory pathways activated by cigarette smoke (53) and reduced inducible nitric oxide synthase (iNOS) concentrations in airway epithelial cells in response to smoking (55). Future studies examining nitric oxide synthesis and reaction products in the bronchial epithelium and submucosa in smokers and non-smokers with asthma should, in parallel, determine extended flow nitric oxide parameters.

A final significant issue that needs to be resolved is the determination of a significant change in any of the flow independent nitric oxide parameters in response to treatment (similar to that which exhaled nitric oxide (measured at 50ml/sec) has been undergoing). This study can be viewed as one of the first steps in addressing this issue. However the recent demonstration that significant changes can be detected in exhaled nitric oxide levels in smokers with asthma with an alternative simple approach (221) may hamper the development of this technique as a replacement method for assessing smokers with asthma.

The inclusion of a group of ex-smokers with asthma in the study has allowed some simple observations to be made. Ex-smokers with asthma have previously demonstrated evidence suggestive of a restoration of corticosteroid sensitivity after many years of smoking cessation (23). The ex-smoking subjects recruited to this study failed to show clear improvements in lung function in response to oral corticosteroids despite a mean duration of quitting of seven years. When compared to smokers and non-smokers with asthma, ex-smokers appeared to represent a separate phenotype with the result for some parameters being equivalent to those observed in smokers and others to the results for non-smokers with asthma. These findings coupled to the observation suggestive of a

restoration of corticosteroid sensitivity in a sub group of ex-smokers with asthma should prompt further research into this group. The proportion of ex-smokers with asthma in the population will substantially increase in coming years due to recent changes in legislation and public perception of smoking and too little is known about the characteristics and behaviour of this group.

Exhaled breath condensate pH has been examined in asthma exacerbations and demonstrates correlation with clinical improvement (57), sputum neutrophilia in COPD and sputum eosinophilia in asthma (238). EBC can be collected outside of the research lab allowing the collection of samples by non-specialised personnel at, for example, the subject's general practice. This facet of EBC may be useful as attendance at hospital based research units may be responsible for some of the reluctance observed in some subjects with regards clinical trial participation. EBC pH has previously been examined by one group in smokers with asthma (58). The authors demonstrated that smokers with asthma had a lower EBC pH than non-smokers with asthma. If this finding was corroborated then the restoration of EBC pH towards the level present in non-smokers with asthma would have provided a suitable exploratory endpoint. In this exploratory study EBC pH measurements were performed at baseline, immediately post oral corticosteroid trial and one month after oral corticosteroids in smokers, ex-smokers and non-smokers with asthma. Surprisingly EBC pH was found to be equivalent in the three groups at all timepoints and therefore unresponsive to high dose oral corticosteroids. The reason for this discordant result is not clear. EBC was collected in this study using a commercially available apparatus and processed in accordance with previously published research. The authors of the previous trial used an unusual method (the subjects breathed through a frozen syringe) for EBC collection and this may have had an effect on the measurements. How this would have a greater effect on the pH of EBC collected from smokers with asthma is not immediately obvious. Another explanation is that both this and the previous trial are small and sampling error may have produced this disparity. Possible additional explanations are that the smokers with asthma recruited to the previous trial may have been experiencing subtle subclinical exacerbations or were not fully recovered from a previous exacerbation and hence would have had a spurious reduction in their EBC pH. The results obtained for this trial leads one to conclude that EBC pH is

insufficiently sensitive and discriminatory to be employed as an exploratory endpoint.

7.5 Conclusion

In conclusion, in the first trial to examine extended flow nitric oxide analysis in a group of smokers with asthma, linear modelling demonstrated that smokers display lower levels of alveolar nitric oxide compared to non-smokers with asthma whilst non-linear modelling demonstrated that alveolar nitric oxide in smokers and non-smokers with asthma was equivalent. Nitric oxide flux and airway wall concentration of nitric oxide are lower in smokers with asthma using both linear and non-linear modelling. Significant differences exist between the result derived for alveolar nitric oxide using linear and non-linear models. The use of extended flow analysis and non-linear modelling may eventually provide a useful exploratory endpoint for the assessment of smokers with asthma but consensus is required with regards the best form of modelling given the lack of agreement between linear and non-linear modelling. Research also needs to be performed to identify the minimal clinically significant change in the parameters derived by extended flow nitric oxide analysis. Finally exhaled breath condensate pH is equivalent in smokers and non-smokers with asthma and does not change in response to a two week oral corticosteroid trial. In light of this finding the utilisation of EBC pH as an exploratory endpoint in clinical trials in smokers with asthma cannot be justified.

8 Conclusions and future directions

8.1 Summary of findings

Smokers with asthma consistently display reduced responses to corticosteroids compared to non-smokers with asthma (5-10, 22-24). This response is associated with worse control of asthma (11, 12), accelerated decline in lung function (15, 21) and increased use of emergency services (16, 17). Smoking is common in asthma with rates reflecting the prevalence in the general population (261) and therefore it represents a significant problem for patients and respiratory physicians.

The results presented in this thesis show that smokers with asthma demonstrate improvements in lung function following treatment with the combination of low dose theophylline and inhaled corticosteroid and treatment with the PPARγ agonist rosiglitazone. Low dose theophylline appears to be acting in synergy with inhaled corticosteroids suggesting the possibility of a re-sensitisation to corticosteroids in smokers with asthma. Low dose theophylline when given alone also improves symptoms in smokers with asthma and should be investigated as a potential alternative treatment for those smokers with asthma not willing to take inhaled corticosteroid. The response to the PPARγ agonist rosiglitazone is the first demonstration of an anti-inflammatory effect through stimulation of this nuclear hormone receptor in humans and may herald a new class of anti-inflammatory agents.

The study attempting to identify mechanisms responsible for the reduced response displayed by smokers with asthma to corticosteroids demonstrated that smokers with asthma display alterations in the pulmonary and systemic cytokine environment suggestive of a deviation from a Th2 to a Th1 inflammatory response. The identification of increased sputum supernatant levels of IL-6 which are resistant to high dose oral corticosteroids is significant given the important role that this cytokine has at the interface between acute and chronic inflammation and the innate and adaptive immune response.

Previous research has suggested that a reduced response to corticosteroids is partially due to an oxidative stress mediated reduction in total HDAC activity through post translational modification in HDAC enzyme isoforms. The results presented here suggest that total HDAC activity in smokers with asthma is equivalent to non-smokers with asthma. However as this is the first attempt to measure this marker in induced sputum technical issues may be preventing the demonstration of a difference and therefore the study should be regarded as non-conclusive.

Smokers with asthma display worse symptoms from asthma when assessed using symptom questionnaires. However their exhaled nitric oxide level measured at the conventional flow rate of 50ml/sec is markedly reduced. Extended flow rate nitric oxide parameters were calculated in smokers with asthma based on the hypothesis that this would reveal increased levels of alveolar nitric oxide correlating with the increased asthma symptoms observe in this group. However smokers with asthma demonstrated lower or equivalent levels of alveolar nitric oxide. This observation combined with the cytokine findings from the same study and the observation that smokers with asthma display reduced response to corticosteroids is consistent with the possibility that inflammation in smokers with asthma is deviated from the eosinophilic/Th2 inflammation displayed by some non-smokers with asthma.

8.2 Limitations of presented research

Conducting a period of original independent clinical research with the aim of obtaining a higher degree requires several compromises. The first and overarching concern is undertaking a study that is feasible within the available time limits. The study examining theophylline and the PPARγ agonist rosiglitazone presented here is short and recruited a small number of subjects and therefore cannot be viewed as the definitive study of these approaches to the treatment of smokers with asthma. However large, multi centre definitive management trials examining these approaches in smokers with asthma can now be conducted as a result of the findings presented here. The study examining potential mechanisms for the reduced response to corticosteroids observed in

smokers with asthma also included a small number of patients and therefore the findings will also require corroboration in larger trials.

Closely allied to the constraints of limited time and finance is the desire to fully interrogate the available data. However to adopt a data mining approach raises the possibility of false positive results due to multiple comparisons. To reduce this possibility each study was conducted according to a prearranged analysis plan and in the case of the trial examining theophylline and rosiglitazone the plan included predefined primary and secondary endpoints. Despite this approach both trials could contain false positive results. Therefore I have presented and discussed the available results with this in mind. All work presented in this thesis should be viewed as exploratory and therefore requiring corroboration.

Another issue of great importance is the characterisation of patients and the differentiation of smokers and ex-smokers with asthma from subjects with COPD. COPD occurs as a direct result of prolonged exposure to inhaled noxious stimuli. In the developed world the agent predominantly responsible is cigarette smoke. Given the common link of smoking and the substantial smoking histories of the recruited subjects it is possible that some of the subjects could actually have COPD. COPD is characterised by chronic airflow obstruction, sputum neutrophilia and a reduced therapeutic response to treatment with corticosteroids (compared to non-smokers with asthma). However despite their substantial smoking histories the recruited smokers and ex-smokers with asthma did not display sputum neutrophilia, developed symptoms at a young age (majority teens to twenties) and also displayed significant bronchodilatory responses to inhaled β_2 agonists. Therefore I feel I can safely argue that the majority of recruited subjects have asthma and not COPD. An alternative view is that I could have recruited a number of subjects who display an overlap in characteristics shared by asthma and COPD. This is certainly possible especially in the older subjects with substantial smoking histories. To address this issue properly I would have had to perform a number of additional screening tests including measurement of transfer factor and high resolution CT scans. Unfortunately this was not feasible due to time and funding constraints. When confirmatory studies are performed to examine the findings presented in this thesis then these issues should be addressed to aid interpretation. The issue of overlap between asthma and COPD

is an important one to address as this combination is regularly observed by the clinician and studies containing well characterised subjects who do display a mixture of the two conditions would provide useful insights for clinical care. This issue coupled to the lack of understanding of the treatment responses of ex-smokers with asthma should stimulate some interesting and clinically relevant future research.

8.3 Conclusions & future directions

Smokers with asthma demonstrate detectable responses to treatment with the combination of low dose theophylline and inhaled corticosteroid, low dose theophylline alone and the PPARγ agonist rosiglitazone. All three treatment combinations should undergo detailed examination in adequately powered management trials in smokers with asthma. The identification of a bronchodilator response to treatment with a PPARγ agonist is intriguing and may herald a new group of anti-inflammatory agents.

Smokers with asthma display altered pulmonary inflammatory conditions compared to non-smokers with asthma and therefore cannot be regarded as equivalent to this group. Further detailed research is required to properly understand the mechanisms responsible for this altered response. Comparison with smokers and ex-smokers with COPD, non-smokers with asthma and normal smoking and non-smoking subjects is required to detail the overlapping and differing patterns of inflammation. Consideration should also be given to the conduct of a large bronchoscopic biopsy study to characterise the histological, immunohistochemical and mechanistic differences between these groups. As smokers with asthma cannot be regarded as equivalent to non-smokers with asthma regulatory bodies should require current and future asthma therapies to demonstrate efficacy in this group as a pre-requisite for licensing. With increased understanding of the pattern of inflammation in this group it is likely that benefits for smokers with asthma and other groups with relative corticosteroid resistance will result.

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