Nasal and whole blood challenge models for allergic rhinitis and COPD

By

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Dedicated to little Noah and his Daddy
Abstract

Nasal allergen challenge (NAC) is a non-invasive model to study the mechanism of allergic rhinitis and effects of novel anti-inflammatory therapies. The repeatability and dose response of a single NAC was studied, followed by development of a daily repeat NAC model involving 4 challenges on consecutive days. However, the results of earlier published work could not be reproduced, since in nasal filter paper (FILT P) eluate in both studies there were no significant changes in levels of interleukin (IL)-4, IL-5 and IL-13. However the daily repeat NAC model did cause priming in terms of symptoms, eosinophils, and nasal lavage IL-5 and IL-13. Proposals were made for implementation of a synthetic absorptive matrix (SAM) as an alternative to Whatman's FILT P and also to deliver higher doses of allergen to the nose. Using SAM resulted in high serial levels of IL-4, IL-5 and IL-13 being detected in SAM eluates during the late phase after NAC (appendix).

In cigarette smokers I requested subjects to exhale cigarette smoke through their noses, to provide a nasal cigarette smoke challenge method. FILT P is likely to distort detected levels of mediators in nasal secretions, but in FILT P eluates there were statistically significant elevations of IL-8 (p=0.01) and IL-12p70 (p=0.02) at baseline in Chronic Obstructive Pulmonary Disease (COPD) patients. However there were increases in nasal lavage MCP-1 at 8h in COPD patients and healthy controls following cigarette smoke (p=0.02).

A cigarette smoke conditioned medium (CSCM) was employed to stimulate whole blood upregulation of CD11b measured by flow cytometry on leukocytes. CSCM caused upregulation of neutrophil CD11b, but this could not be inhibited by anti-oxidants. In contrast, menadione (MQ) stimulates intracellular generation of oxidants, and this caused neutrophil CD11b upregulation that was inhibited by glutathione. Xanthine with xanthine oxidase (X+XO) generates an extracellular source of oxidants, and this caused an increase in neutrophil CD11b on washed blood cells, that could also be inhibited by glutathione.

NAC or cigarette smoke nasal challenge, as well as stimulation of human whole blood with oxidants, have potential as challenge models - but SAM should be used instead of FILT P.
Statement of Contribution

For the single dose nasal allergen challenge (NAC) repeatability and dose response study I assisted in the design of the study, and carried out the clinical phase with Dr. Edward Erin and nursing and technical staff on the CSU. Dr Edward Erin carried out the Luminex analysis of cytokines and chemokines. I carried out independent single-handed data processing, with generation of summary statistics and production of figures and tables.

For the repeat (4 consecutive days) dose nasal allergen challenge (NAC) study I took part in the design of the study, protocol generation and Ethics Committee submission, and carried out the entire clinical phase with nursing and technical staff on the CSU. Dr Edward Erin carried out the cell counts of the nasal lavage cytospins. I and Mr Grant Nicholson carried out the Luminex analysis of cytokines and chemokines of the filter paper eluates. The cytokine and chemokine analysis of the nasal lavage samples were carried out by Zarin Brown and Jennifer Willis of Novartis. I carried out independent single handed data processing, with generation of summary statistics and production of figures and tables.

For the cigarette smoke nasal challenge study I took part in the design of the study, protocol generation and Ethics Committee submission, and carried out the entire clinical phase with nursing and technical staff on the CSU. I carried out the cell count of nasal lavage, Luminex analyses of the nasal filter paper eluates. I and Mr Grant Nicholson carried out the Luminex analysis of cytokines and chemokines of the filter paper eluates. The cytokine and chemokine analysis of the nasal lavage samples were carried out by Zarin Brown and Jennifer Willis of Novartis. I carried out independent single handed data
processing, with generation of summary statistics and production of figures and tables.

For the whole blood stimulation with CSCM and oxidants I designed the experiments and independently carried out the stimulation and flow cytometry. I carried out independent single handed data processing, with generation of summary statistics and production of figures and tables.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AHR</td>
<td>Airways hyperreactivity</td>
</tr>
<tr>
<td>ARIA</td>
<td>Allergic Rhinitis and its Impact on Asthma</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C3a</td>
<td>Complement component C3a</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CSCM</td>
<td>Cigarette smoke conditioned medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>EDN</td>
<td>Eosinophil derived neurotoxin</td>
</tr>
<tr>
<td>ELF</td>
<td>Epithelial lining fluid</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FILT P</td>
<td>Whatman's no 42 filter paper</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GAFS</td>
<td>Gated Autofluorescence and Forward Scatter</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative on Asthma</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative on chronic Obstructive Lung Disease</td>
</tr>
<tr>
<td>GRO-α</td>
<td>Growth-regulated oncogene α</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HOCL</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-γ inducible protein 10</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting β₂-adrenoceptor agonist</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄</td>
</tr>
<tr>
<td>MAP-K</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence activity</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Murine macrophage inflammatory protein 1 alpha</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Mo-AB</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MQ</td>
<td>Menadione</td>
</tr>
<tr>
<td>NAC</td>
<td>Nasal allergen challenge</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>Nitrate</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PGD₂</td>
<td>Prostaglandin D₂</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI media</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>SAM</td>
<td>Synthetic absorptive matrix = Accuwik Ultra</td>
</tr>
<tr>
<td>SCCA</td>
<td>Squamous cell carcinoma antigens</td>
</tr>
<tr>
<td>SLIT</td>
<td>Sublingual immunotherapy</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TAME</td>
<td>Na-p-tosyl-L-arginine methyl esterase</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>X</td>
<td>Xanthine</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
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1 Introduction

1.1 Clinical aspects of allergic rhinitis, asthma and COPD

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are diseases that cause a large global burden of disease, with significant amounts of morbidity and mortality. Both asthma and COPD commonly present to General Practitioners and Respiratory Physicians. Allergic rhinitis is a common but generally mild condition, that nevertheless may cause considerable discomfort.

1.1.1 Allergic rhinitis

Allergic rhinitis is defined by the Allergic Rhinitis and its Impact on Asthma (ARIA) work group as “a symptomatic disorder of the nose induced after allergen exposure due to an IgE-mediated inflammation of the membranes lining the nose (1).” Allergic rhinitis is characterised by sneezing, nasal itching, nasal obstruction and mucous discharge. In addition patients can feel systemically unwell and suffer additional symptoms such as headaches, watery itchy eyes and skin rashes. If the rhinitis is severe it can cause disease of the paranasal sinuses causing pain or dysfunction of the Eustachian tube with resultant ear symptoms (2) Over 500 million patients worldwide suffer with allergic rhinitis (3) Allergic rhinitis is common in young working adults and can cause poor sleep, interruption of daily activities and can increase the severity of associated asthma. Allergic rhinitis has been shown to reduce attendance at school and work (4;5) and in addition to the demand for access to medical treatment this is associated with a significant economic burden (6).
1.1.2 Asthma

Asthma is a disease of variable airflow obstruction and is characterised clinically by variable cough, chest tightness and wheeze (7). Most patients are well between episodes although there is a significant minority who continue to have symptoms despite maximal therapy (8). Asthma is one of the most common diseases, with the World Health Organisation (WHO) estimating that globally there are 155 million asthma sufferers, affecting all ages in both high and low income countries (9). A review of the General Practice Research Database has shown that during the 1990's the prevalence of asthma in UK primary care was increasing (10). Studies have shown that asthma is associated with a significant reduction in health related quality of life (11-14). Among patients with asthma work limitation is common (15-17). Asthma has important social relevance as it affects school age children and hence can affect their educational progress, and also it occurs in young adults who form an important part of the workforce and can lead to significant economic costs (18;19).

1.1.3 COPD

COPD is a common disease caused almost exclusively by smoking in industrialised societies, however only 10-20% of smokers develop COPD. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has formulated a “Global Strategy for the Diagnosis, Management and Prevention of COPD”, (www.goldcopd.com) that is regularly updated (20).

A working definition of COPD is given within the GOLD Global Strategy (2007) as: “A preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterised by airflow limitation that is not fully reversible. The
airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases." Clinical features of COPD include cough, sputum production, wheeze and breathlessness. Pathology distant from the lung also occurs and includes right heart failure, systemic disease with cachexia and respiratory and peripheral muscle weakness (21). COPD is an important disease globally and the morbidity and mortality continue to rise with COPD predicted to become the third commonest cause of death by 2020(22) COPD is associated with a significant reduction in health-related quality of life (23-26). Whilst COPD generally affects the older population COPD it does still affect people of working age (27), and leads to work disability (28).

The obstructive lung defect typical of COPD is a decreased FEV₁ compared to that predicted for an individual (29). The FEV₁ should be measured following the inhalation of a bronchodilator and expressed as a percentage of that predicted for an individual (30).

The exact burden of COPD is difficult to quantify as misdiagnosis and under diagnosis is common. A review of the General Practice Research Data Database estimates that nearly 900,000 patients in the United Kingdom have COPD (31), and COPD was recorded as the principal cause of death in 23,204 people in 2004 (32). This makes COPD the fifth most common cause of death after ischaemic heart disease, stroke, lung cancer and pneumonia. COPD is also a leading cause of morbidity and mortality worldwide (33), and in addition the prevalence of COPD is increasing worldwide.
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1.2 Molecular and cellular pathophysiology of allergic rhinitis, asthma and COPD

1.2.1 Allergic rhinitis and asthma

The immunopathology of allergic disease is complex and the clinical disease entity is a result of interaction between a variety of stromal and immune cells involving an array of inflammatory mediators. The pathogenesis of allergy and asthma as been the subject of several reviews in the literature from 2007 and 2008 (34-38), and they all emphasise that a single mechanism cannot explain the clinical diversity of the disease. The hypothesis of allergen-triggered Th2 stimulation and resultant inflammation is too simplistic, as illustrated by the way that cyclosporine has been disappointing as a therapy for severe asthma, indicating that the T cell may not be essential for the development of asthma. The role of the eosinophil has traditionally been thought to be pivotal to the development of asthma, however clinical trials of anti-IL-5 have shown profound reductions in peripheral blood eosinophil levels but have again produced disappointing clinical effects. It has been noted that subjects with more severe asthma that is resistant to steroids may have predominantly neutrophilic inflammation (39).

The following description of the pathogenesis of allergic inflammation demonstrates that there are many potential mechanisms that can result in the clinical features of asthma. In addition, the genetic basis of asthma is not straightforward, with over 100 genes potentially involved, illustrating the complexity of gene-environment interactions in causing asthma phenotypes. Asthma is clearly a heterogeneous disease as evidenced by differing clinical presentations (e.g. allergic, aspirin-sensitive, exercise-induced, severe asthma...
etc.) and differing responses to treatments. Gary Anderson has published a thought-provoking recent review that has suggested that individual patients could be characterised and treatments tailored to the specific inflammation causing the majority of their symptoms, so called “endotyping of asthma”(38).

1.2.1.1 Mast cells, eosinophils and changes in the early phase

There has been interest in the pathological basis of allergic disease for over a hundred years (40). In 1879 Ehrlich first described mast cells and eosinophils, by performing microscopy on stained leukocytes (41). The description of IgE binding to mast cells and causing degranulation and hence mediator release was described by Johansson in 1967 (42). Mast cell degranulation is triggered by the binding of IgE to Fcε1 (43). The products of mast cell activation include the release of granule contents including histamine, heparin and β-tryptase and the synthesis of inflammatory mediators such as prostaglandin D2 (PGD2) and leukotrienes.

Allergen challenge of allergic individuals has been shown to increase levels of histamine and tryptase in nasal lavage fluid, and these changes are not seen in non-allergic subjects (44). Nasal allergen challenge (NAC) has also been noted to increase levels of histamine in nasal lavage fluid in a time and dose dependent manner (45). PGD2 is produced by mast cells and been detected in high concentrations at the site of allergic inflammation (46;47). β-Tryptase is a serine protease that is released from degranulating mast cells and has inflammatory actions through PAR-2 activation (48). In addition tryptase may also contribute to airway oedema through microvascular leakage (49). In a NAC study a reversible β-tryptase and trypsin inhibitor has been shown to reduce symptoms, eosinophils, and levels of IL-5 following NAC (50). Nasal challenge
with ragweed antigen causes a rise in complement activation products in allergic but not normal subjects that correlated closely with symptoms, suggesting a role for complement activation in the early phase of the allergic response (51).

Eosinophils are leukocytes of bone marrow origin that migrate via the bloodstream to mucosal sites. Eosinophils contain highly basic granules that give these cells their characteristic staining properties, and the granules can be released to combat parasitic infections (52). The eosinophil granule proteins include major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN).

Of particular relevance to asthma is that MBP is prominent in the sputum and mucosal tissue of asthmatics, and can cause airways hyperreactivity (AHR) (53). GM-CSF, IL-3 and IL-5 are cytokines that enable eosinophil survival, acting via a common shared beta-chain (CD131) (52). Eosinophils are thus potentially long-lived tissue cells, which can produce cytokines (notably TGF-β), present antigens, and interact with fibroblasts and participate in airways tissue remodelling (54).

Eosinophils are also involved in the fibrotic reaction that occurs in allergic inflammation. The tenascins are a group of glycoproteins found in the extracellular matrix that are important in normal development and can be associated with pathology (55). In a skin challenge model eosinophil-derived factors that promote fibrosis (TGF-β and IL-13) were associated with tenascin and procollagen-I immunoreactivity (56). Interleukin (IL)-5 is of crucial importance for eosinophilic inflammation, since it is the critical cytokine involved in eosinopoiesis in the bone marrow. A blocking antibody to IL-5 depletes
eosinophils from the circulation and sputum of asthmatic patients but, disappointingly, had no effect on the response to inhaled allergen, airway hyper-responsiveness symptoms, lung function or exacerbation frequency in asthmatic patients (57). Furthermore, 2 larger studies in patients with symptomatic asthma have demonstrated only minor effects of anti-IL-5 on lung function and symptoms in asthma (58). However, IL-5 may play a role in airway remodelling as anti-IL-5 reduces eosinophil infiltration and tenascin deposition in a skin model of allergen challenge (59).

Neural mechanisms in allergic disease have been investigated. Nerve growth factor (NGF) enhances the production of substance P by neurons (60). Substance P is known to trigger smooth muscle constriction and acute inflammation (61;62). Neurotropins such as NGF seem to be the link between immune and neural mechanisms of inflammation (63). Using capsaicin to stimulate nerves has shown that nonallergic rhinitis is not characterised by increased responsiveness of capsacin-sensitive nerve fibres in contrast to allergic rhinitis where there is marked hyperresponsiveness (64). Hypertonic saline has been shown to cause nasal inflammation via a neural pathway and subjects with allergic rhinitis have an enhanced response involving capsicacin-sensitive fibres, consistent with nasal hyperresponsiveness (65). A murine model has demonstrated that NGF augments the early phase allergic response through increased release of mast cell mediators (66).

1.2.1.2 Epithelium and dendritic cells- T-cell recruitment and late symptoms

The nasal epithelium is rich in dendritic cells, which are antigen-presenting cells with the ability to induce primary immune responses. Dendritic cells can prime T
helper cells directly although the exact mechanisms involved are not clear (67). Increased levels of mucosal dendritic cells are seen in both allergic rhinitis and asthma, and studies of blood derived dendritic cells have shown differences in the function of dendritic cells between atopic and normal subjects (68).

T helper cells (CD4+) can be divided into 2 groups, Th1 and Th2, based on their profile of cytokine production. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th1 cells produce IL-2, IFN-γ, and TNF-β (69). The factors that determine which group of T helper cells are pre-dominant have been extensively studied, but not yet fully elucidated, although it is clear that it is a combination of genetic and environmental factors (70). The regulation of IgE production by B cells by cytokines produced by T-cell derived cytokines was initially described in mice (71) and was subsequently described in humans (72). It has long been known that there is an association between BAL T-cell activation, eosinophilia and asthma severity (73), and deactivation occurs following steroid treatment (74). A study of nasal biopsies following nasal allergen challenge has shown significantly increased numbers of activated T helper cells (75).

The evidence suggests that allergen exposure causes Th2 recruitment and/or differentiation of these specific T cells. These Th2 cells then produce IL-4 and IL-13 which induces IgE production from B cells (72). Degranulation of IgE-coated mast cells causes the acute changes seen in the early phase. IL-5 production by the Th2 cells would activate eosinophils and contribute to the late phase reaction (76). It has been shown that PGD₂ stimulates the production of IL-4, IL-5 and IL-13 by human Th2 cells at levels found at the site of allergic inflammation, and gene transcription peaks at 2 hours (77).
Several studies have shown that interferon (IFN)-γ is increased in asthmatics and that Th1 activation occurs (78-80). It has also been shown that IFN-γ-producing cells and IFN-γ levels are increased during exacerbations of asthma (81;82). Th1 activation can increase bronchial hyperreactivity once allergic disease is established (76). Th1 activation occurs in the more severe forms of allergic disease including asthma, rhinitis and atopic dermatitis (82-85).

Another set of CD4+ cells, known as Th17 cells, have recently been described that have an important role in inflammatory disease (86;87). Th17 cells produce IL-17 and increased concentrations of this cytokine have detected in the sputum of patients with asthma (88). IL-17 has been shown to promote neutrophilic inflammation by inducing the release of pro-inflammatory cytokines from airway epithelial cells (89). It is hypothesised that Th17 cells are important in the development of the neutrophilic infiltration that occurs in more severe corticosteroid dependent asthma (90). It is now recognised that that specific cytokines can promote transcriptional factors such as T-bet, GATA3, RORγt, and Foxp3 that influence Th1, Th2, Th17, and Treg (T régulator) cells, respectively (91). CD4+ T cells can also differentiate into a group of cells labelled as Tregs that act to suppress T-cell responses (92), FOXP3 is a transcription factor that is responsible for the suppressive functions of Tregs. TGF-β can also induce the transformation of naive CD4+ cells to become Tregs and are classified as inducible Tregs (93).

The production of TH2-type cytokines such as IL-4, IL-5 and IL-13 are characteristic of allergic diseases. A study of nasal biopsies at 6 hours following nasal allergen challenge has shown that the levels of eosinophils but not T-cells increase in nasal mucosa. Co-localisation studies showed that the majority of
the IL-4 and IL-5 mRNA was associated with eosinophils (94). This is important because it suggests that eosinophils are the source of early phase cytokines that lead to the development of a late phase response. IL-5 levels have also been shown to increase in nasal filter paper samples during the early and late phase following nasal allergen challenge; however IL-4 was not detected in this study (95). Another biopsy study has shown that NAC induces a significant tissue eosinophilia with an associated increase in IL-8, IL-13 and RANTES mRNA-positive cells and that increased levels of IL-13 mRNA-positive cells are still present 1 week following single NAC (96). A co-localisation study has determined that the majority of RANTES mRNA-positive cells following NAC are macrophages, followed by eosinophils (97). It has been shown that following NAC there are parallel increases in eosinophil counts, eosinophil protein X, and eotaxin concentrations in nasal lavage fluid (98).

NAC has been shown to increase levels of the cytokines IL-4, IL-5 and IL-13 in filter paper samples collected during the late phase. The chemokines eotaxin, RANTES, MCP-1, MIP-1α, IL-8 and IP-10 were also produced during the late phase. It has been shown in a guinea pig model of allergic rhinitis using an anti-IL-5 antibody that IL-5 is essential for eosinophil migration from the bone marrow to the nasal airway; neither IL-5 nor eosinophils are required for the development of nasal symptoms and nasal hyperresponsiveness (99). Interestingly levels of TH1 cytokines (IL-2, IL-3, IL-7, IL-12, IL-15, TNF-α, IFN-γ, GM-CSF) were not affected (100). It has also been shown that a single dose of nasal steroid has the ability to abolish the IL-5 and IL-13 responses following nasal allergen challenge (101).
Inhalation of IL-4 increases levels of airways eosinophils (102), while intranasal administration of IL-4 to non-allergic individuals increased levels of IL-6, eosinophils, and neutrophils in nasal lavage fluid 24 hours following challenge (103).

Changes to the epithelium in allergic rhinitis remains a controversial area. One study has shown that there is no evidence of epithelial damage (104). An electron microscopy study in perennial rhinitis has shown increased numbers of epithelial cells with cytoplasmic vacuoles and markedly widened intercellular spaces, changes which are not visible on light microscopy (105). This break in integrity of the epithelial barrier may allow allergen to reach the subepithelial layers. In addition to the epithelial changes there has been shown to be thickening of the basement membrane (106). The protein filaggrin is involved in maintaining the tight junction between epithelial cells, and a genetic variants with abnormalities of this protein suffer from atopic dermatitis and asthma (107).

Airway remodelling is seen in asthma and comprises epithelial cell mucus metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis, and increased angiogenesis (108). Several cytokines have been implicated in this process including TGF-β, VEGF, Th2 cytokines (IL-5, IL-9, IL-13) and NF-κB regulated cytokines (109). As described below there are differences in the histology of the nasal mucosa compared to the respiratory mucosa and the relevance of airway remodelling in allergic rhinitis has not been extensively studied, and it has been suggested that the differences in remodelling between allergic rhinitis and asthma may be due to the lack of smooth muscle in the nasal mucosa or their differing embryological origins (110).
Figure 1.1 shows how the inflammatory mechanisms above combine to produce the features of the early and late phase allergic response.

![Inflammatory mechanisms in the allergic response](image)

**1.2.2 Comparison of allergic rhinitis and asthma**

The respiratory mucosa of the nose has many similarities to that of the bronchi. It consists of a pseudostratified columnar ciliated epithelium resting on a basement membrane under which there are vessels, mucous glands, fibroblasts, inflammatory cells and nerves. However there are differences, since in the nasal mucosa the blood vessels are much more prominent and there is an absence of airway smooth muscle (111). It has been noted that nasal symptoms frequently co-exist in patients with asthma (112), and review of epidemiological data shows that asthma and rhinitis often co-exist in the same patient (113).

Patients with allergic rhinitis have increased bronchial sensitivity to methacholine or histamine especially during and slightly after the pollen season.
(114;115), and there is a two-way relationship between nasal and bronchial inflammation. It has been shown that bronchial allergen challenge leads to increases in nasal eosinophils and enhanced expression of IL-5 in nasal epithelium (116). Patients with allergic rhinitis with no history of asthma develop bronchoconstriction after endobronchial allergen challenge with associated inflammation (117;118). There is considerable similarity between the inflammation that occurs in allergic rhinitis with that occurring in asthma. Examination of the bronchial mucosa in patients with allergic rhinitis shows eosinophilic inflammation (119). Increased numbers of mast cells have been seen in the both the nasal and bronchial mucosa in allergic disease (120;121). Increased levels of Th2 lymphocytes have also been shown in both disease states (122;123). The response following allergen challenge has an early and a late phase response in both the nose and the lungs (85;(124-126). One of the main differences in allergic rhinitis is that the lack of smooth muscle may affect airway remodelling. It has been shown that airway remodelling does - occur in rhinitis although this is much more pronounced in asthma (127).

It can be seen that there is epidemiological and pathological evidence that allergic rhinitis and asthma are closely related; and hence it is plausible to suggest that using an allergen challenge model of the nose would be a useful surrogate to test potential new anti-inflammatory therapies for asthma without having to carry out inhaled allergen challenge nor bronchoscopy with their associated risks.

1.2.3 COPD

1.2.3.1 Cigarette smoke composition

Cigarette smoke has been estimated to contain some $10^{17}$ oxidant molecules per inhalation, and there is considerable evidence that oxidative stress is
increased in patients with COPD (128-132). The lungs have a large epithelial surface area that is exposed to reactive oxygen and nitrogen species (ROS and RNS) within cigarette smoke, further oxidants are generated during inflammatory processes, and an oxidant/antioxidant balance is thought to be important in the pathogenesis of COPD (133). Cigarette smoke is a heterogeneous aerosol generated by incomplete burning of tobacco leaves. It is composed of gaseous, volatile and particulate components with up to 95% of the weight of smoke in the gaseous phase. More than 400 substances are present in cigarette smoke including oxidants; pharmacologically active compounds such as nicotine, mutagens and carcinogens; as well as antigenic and cytotoxic components. Nicotine is a toxic plant alkaloid that stimulates acetylcholine receptors at autonomic ganglia of both the sympathetic and parasympathetic systems as well as having stimulant activities at neuromuscular receptor sites and in the central nervous system. Carbon monoxide interferes with oxygen transport and utilisation due to formation of carboxyhaemoglobin (COHb). Carcinogens include aromatic hydrocarbons, amines, and nitrosamines.

Both the gas phase of cigarette smoke and the tar component can lead to the generation of ROS (134-136) (Figure 1.2). In addition to direct inhalation of ROS from cigarette smoke, the inflammatory process that occurs in smoker's lungs can lead to further generation of oxidants by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in leukocytes drawn to the site of inflammation. Neutrophil death and necrosis leads to high concentrations of neutrophil elastase and ROS in sputum (137). ROS may also be important mediators in asthma (138).
Reactive oxygen species (ROS) include superoxide anion (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (·OH). Most of the oxygen we inspire is reduced within the mitochondrial electron transport chain in a reaction catalysed by cytochrome oxidase to produce water (133). To allow continued electron transport and ATP generation, cytochrome oxidase donates its electron to oxygen to generate ROS such as O$_2^{-}$. In addition, O$_2^{-}$ is generated by NADPH oxidase found in neutrophils and monocytes/macrophages. O$_2^{-}$ is relatively unstable and does not readily cross cell membranes. O$_2^{-}$ may be converted to H$_2$O$_2$ by superoxide dismutase (SOD), and the H$_2$O$_2$ may itself be oxidised by myeloperoxidase (MPO) or eosinophil peroxidase (EPO) to hypochlorous acid (HOCI). Alternatively, O$_2^{-}$ and H$_2$O$_2$ may interact in the presence of ferrous ions (Fe$^{2+}$) to form the highly reactive ‘OH, which is thought to be a major mediator of ROS damage.

Free radicals can also react with polyunsaturated fatty acid side chains that are present in membrane or lipoproteins, leading to the formation of lipid peroxidation products such as isoprostane which is a marker of oxidant stress but may have important effects as a mediator of disease (139). Oxidants also activate the transcription factor for NF-κB which then promotes the transcription of many other pro-inflammatory mediators, while hydrogen peroxide causes an increase in histone acetylation (140). Heme oxygenase-1 (HO-1) is a stress response protein induced by oxidants (141).

Reactive nitrogen species (RNS) include nitric oxide (NO) and its derivatives such as nitrogen dioxide (NO$_2$) and peroxynitrite (ONOO$^-$) (142) (Figure 1.3). NO is itself a gaseous radical and has complex regulatory actions in a variety of inflammatory and infectious conditions. Nitric oxide synthase (NOS) converts L-
arginine to L-citrulline and generates NO. $O_2^-$ can react with NO to form ONOO$, a very unstable and highly reactive molecule that mediates many of the damaging effects of oxidative stress (142). When ONOO$ becomes protonated it isomerises to trans-peroxy nitrous acid, which leads to the formation of $'OH. ONOO$ reacts with tyrosine residues within certain proteins to form stable 3-nitrotyrosines which may be detected immunologically. Increased levels of 3-nitrotyrosines have been detected in sputum macrophages from patients with COPD (143). Nitrite ($N_2O_2^-$) is a major end product of NO metabolism that is rapidly metabolised to nitrate ($NO_3^-$) and has been shown to promote tyrosine nitration by reaction with hypochlorous acid (HOCl). Myeloperoxidase has the capacity to produce nitrating oxidants and may also nitrate tyrosine residues (144;145).

**Figure 1-2 Generation of oxygen radicals from cigarette smoke**
Nitric oxide (·NO) metabolism

Nitrogen dioxide radical

\[ \text{HO}^\cdot + \text{O}_2^\cdot \rightarrow \text{ROO}^\cdot \rightarrow \text{NO} \]

Figure 1-3 – Generation of nitrogen radicals from cigarette smoke

1.2.3.2 Oxidant stress in COPD

The lungs are unique because they have a large epithelial surface area that is exposed to oxidants or generators of oxidant stress such as cigarette smoke or airborne pollutants. There is considerable evidence that oxidative stress is increased in patients with COPD (128-130;132;146). The major oxidants in airways are reactive oxygen and reactive nitrogen species (ROS/RNS). ROS include superoxide, hydrogen peroxide and hydroxyl radicals. RNS include nitric oxide and its derivatives such as nitrogen dioxide and peroxynitrite. Each puff of cigarette smoke contains in the order of \(10^{17}\) ROS molecules. In addition to direct inhalation of ROS from cigarette smoke, the inflammatory processes that
occur in smokers lungs can lead to further generation of oxidants by nicotinamide adenine dinucleotide phosphate (NADPH) in leukocytes drawn to the site of inflammation. Neutrophil death and necrosis leads to high concentrations of neutrophil elastase and ROS in sputum (137).

Superoxide radicals can combine with nitric oxide to form peroxynitrite; this is a very stable molecule which increases its ability to diffuse through a cell to find a target. When peroxynitrite becomes protonated it isomerises to trans-peroxy nitrous acid, which leads to the formation of hydroxyl radicals, a further oxidant stress. Peroxynitrite reacts with tyrosine residues within certain proteins to form 3-nitrotyrosines which may be detected immunologically. Increased levels of 3-nitrotyrosines have been detected in sputum macrophages from patients with COPD (143). Nitrite is a major end product of nitric oxide metabolism, and has been shown to promote tyrosine nitration by reaction with the inflammatory mediators hypochlorous acid or myeloperoxidase an enzyme found in neutrophils (144).

Assessment of oxidant and antioxidant status may be performed in a non-invasive manner by analysis of exhaled breath condensate (147-152). Markers of oxidative stress measured in exhaled breath of patients with COPD include 8-isoprostane (153;154), ethane (155;156) and aldehydes (157). There is an increase in these markers during exacerbations (154;158). However, concerns have been expressed about the reproducibility of some of these oxidative stress biomarkers assessed in breath condensate, sometimes due to sensitivity of assays in relation to low concentrations (159-161). A problem with measurement of exhaled hydrogen peroxide in COPD is that there must be a properly matched control group with matched smoking status and age
Markers of oxidative stress are also increased in smokers with normal lung function, but to a lesser extent than in COPD patients (151).

The lungs have protective antioxidant mechanisms, and damage can occur when this equilibrium is disturbed either by depletion of antioxidants or excess production of ROS or RNS (133). Superoxide dismutase (SOD) acts to protect the lungs from increased oxidative stress by catalyzing the formation of H2O2, which is then degraded by catalase to water and oxygen (163). Glutathione (GSH) is a ubiquitous tripeptide that contains a sulphydryl (-SH) group that enables it to protect cells against oxidants (164;165). Oxidant stress induces glutathione synthesis in human bronchial epithelial cells (166). H2O2 can be rendered less toxic by reacting with reduced glutathione under the action of glutathione peroxidase. Glutathione peroxidase is present within cells and is also secreted into the epithelial lining fluid. It has been shown that bronchial epithelial cells, alveolar macrophages and interstitial cells are potential sources of pulmonary extracellular glutathione peroxidase (167). Nonenzymatic antioxidants also exist; these include vitamin E, vitamin C, β-carotene, uric acid, glucose, bilirubin, taurine, albumin, and cysteine and cysteamine.

It has been shown that plasma antioxidant capacity is significantly reduced in healthy smokers and in patients with COPD compared with non-smoking healthy subjects (168). These changes were present in patients with COPD who were ex-smokers suggesting that at least a component of the oxidative stress comes from endogenous inflammation rather than the direct effects of cigarette smoke. Sputum concentrations of glutathione disulphide (GSSG) and nitrosothiols are increased in sputum supernatants of patients with COPD (169). The ability of epithelial lining fluid (ELF) to provide antioxidant protection to lung
parenchymal cells has been evaluated by assessing the response of cells in culture in response to hydrogen peroxide (170). It was demonstrated that ELF does provide protection against H$_2$O$_2$, and this is mainly due to the presence of catalase. Oxidants cause a variety of cell signalling events including activation of adenylate cyclase, IκB kinases and NF-κB, p38 MAPK and PI-3κγ. These cause inflammatory gene activation and release an upregulation of cytokines, chemokines, adhesion molecules and proteases.

### 1.2.3.3 Inflammatory cells in COPD

**Neutrophils**

Neutrophils have been found in increased numbers in the sputum and BAL fluid of patients with COPD (171-175). An increased level of sputum neutrophils is associated with a rapid decline in FEV$_1$ in smokers, indicating that neutrophils may play a role in disease progression (176). It is also interesting to note that induced sputum from ex-smokers show similar neutrophil levels suggesting that inflammation may persist once established (176-178). However increases levels of neutrophils are not seen in bronchial biopsies from patients with COPD (171;179;180). This may be due to the neutrophils being in a location other than the subepithelial zone obtained in biopsies or rapid transit of neutrophils through the lung parenchyma into the lumen. There are a number of chemotactic stimuli that may lead to neutrophil accumulation including LTB$_4$ and IL-8 and increased levels of IL-8 have been found in the BAL fluid of smokers susceptible to COPD (181). Neutrophil recruitment to the airways involves vascular adhesion and E-selectin has been shown to be upregulated on the bronchial epithelium in COPD (182).
Neutrophils secrete serine proteases including neutrophil elastase, cathepsin G, and proteinase-3, as well as matrix metalloproteinase (MMP) 8 and MMP-9. The observation that increased neutrophils are not found within biopsy specimens is consistent with the observation that there is a negative correlation between number of neutrophils and amount of alveolar destruction (183). These enzymes are however potent mucus stimulants and excess mucus production is a feature of COPD.

Macrophages

Macrophages are thought to play a significant role in the development of COPD. Macrophage numbers are increased in BAL fluid of patients with COPD (184), and numbers of macrophages in the airways correlates with the amount of parenchymal destruction in emphysema (179). In addition macrophages are concentrated in the centriacinar zones where emphysema is most marked (179). The number of macrophages also correlated with disease severity (185). Increased levels of MCP-1 and GROα have been found in the sputum and BAL fluid of COPD patients providing a mechanism for macrophage recruitment (186;187). Macrophages release inflammatory mediators and elastolytic enzymes when stimulated with CSCM in vitro and this provides a mechanism for the inflammatory changes seen in COPD (188-190).

T lymphocytes

Increased numbers of CD45+ cells (total leukocytes) and CD3+ cells (T lymphocytes are found in chronic bronchitis with a greater proportional increase in CD8+ (cytotoxic/suppressor) cells (191-193). The increase in CD8+ cells is associated with a decline in lung function (180). It is not yet clear how T cells are attracted to the lung although it has been suggested that there is chronic
immune stimulation via antigens presented via the HLA class 1 pathway (194). It also remains unclear how the CD8+ cells contribute to the pathological features of COPD, although their ability to cause apoptosis of alveolar cells may play a role (195; 196).

Epithelial cells

In normal lung the airway epithelial cells act in a protective manner by producing anti-oxidants, defensins and anti-proteinases. However airway epithelial cells may be important in the initiation and propagation of inflammation in COPD as they are stimulated by cigarette smoke to produce TNF-α, IL-1β, GM-CSF and IL-8 (197-199).

Eosinophils

Whilst eosinophils are characteristically thought to be a feature of allergic disease there is some evidence for their role in the pathogenesis of COPD, although this remains controversial. It has been noted that the presence of eosinophils in the sputum predicts a response to treatment with corticosteroids, and it has been proposed that this indicates co-existing asthma (200). It has been noted that there are increased numbers of eosinophils present during acute exacerbations of COPD and this is due to upregulation of RANTES in the epithelium and sub epithelium (201).

1.2.3.4 Cytokines and chemokines

Increased levels of IL-6, IL-1β, TNF-α and IL-8 have been found in sputum samples from patients with stable COPD (174). Levels of TNF-α, IL-1β, IL-6, IL-8 and MCP-1 are increased in BAL taken from smokers compared with non-smokers (202). The CC chemokine macrophage chemotactic peptide-1, MCP-1,
is increased in BAL of COPD patients (187). MCP-1 is a potent chemoattractant for monocytes, and acts via CCR2. MCP-1 causes T-cell and monocyte migration, and this may lead to the accumulation of these cells into the airways in COPD. GRO-α (growth related oncogene-α) is elevated in COPD (186) and is chemotactic for monocytes as well as neutrophils and may therefore contribute to the increased numbers of macrophages that are derived from blood monocytes in COPD. Elevated expression of MCP-1, TGF-β and IL-8 and their mRNA has been seen in the bronchiolar epithelium in smoker with COPD compared with smokers without COPD (203).

There are increased amounts of LTB4, a potent chemoattractant of neutrophils, in the sputum of patients with COPD (204). LTB4 is probably derived from alveolar macrophages and neutrophils themselves. Alveolar macrophages from patients with α1-anti-trypsin deficiency secrete greater amounts of LTB4 (205).

IL-8 is a chemoattractant for neutrophils present in high concentrations in induced sputum of patients with COPD (174;206), and found in increased amounts in BAL fluid (206;207) IL-8 is secreted by macrophages, neutrophils and by airway epithelial cells (208) and signals through two receptors: a low affinity CXCR1 specific for IL-8 that is involved in neutrophil activation, and a high affinity CXCR2 that is activated by a range of CXC chemokines including IL-8, growth related oncogene (GRO-α, -β, -γ), and epithelial-derived neutrophil activating peptide, ENA-78. CXCR2 is involved in the chemotaxis of neutrophils and monocytes. Higher levels of IL-6 and IL-8 are found in the induced sputum from patients with increased numbers of exacerbations (209).

TNFα is present in high concentration in the sputum of COPD patients (174), especially during exacerbations (210). TNFα may activate the transcription of
NF-κB, which switches on the transcription of inflammatory genes, including chemokine (IL-8) genes and proteases, in macrophages and epithelial cells (211). Serum concentrations of TNFα and stimulated TNFα production from peripheral blood monocytes are increased in weight losing COPD patients (212).

Transforming growth factor-β1 has increased expression in small airway and alveolar epithelial cells in COPD, and participates in the fibrotic processes that take place in the small airways (213;214). As fibrosis has been shown to be a component of COPD pathogenesis then extrapolation of the processes that occur in fibrotic lung disease may be relevant. TGF-β, connective tissue growth factor, platelet-derived growth factor and IL-13 have all been shown to be involved in the pathogenesis of idiopathic pulmonary fibrosis (215). IL-13 and interferon-γ are overexpressed in a murine form of emphysema that is mediated by increased expression of MMPs and cathepsins (216;217). Endothelin-1 levels are increased in the sputum of patients with COPD, particularly during exacerbations (218;219). ET-1 may be important in pulmonary vascular remodelling in severe pulmonary hypertension (220). Cigarette smoke extract has been shown to induce COX-2 with concurrent synthesis of prostaglandin E₂ by normal human lung fibroblasts producing a pro-inflammatory environment (221).

1.2.3.5 Protease-Antiprotease Balance

The key to the pathogenesis of emphysema is believed to be an imbalance of proteases and antiproteases in the lung (222). An excess of proteases capable of degrading extra-cellular matrix components, in comparison to their inhibitors, leads to the destruction of the extracellular matrix seen in emphysema. Elastin
degradation may be particularly important in the pathogenesis of emphysema since elastin cannot be regenerated in an active form (223).

Proteases are also potent stimulators of mucus secretion. Neutrophils and macrophages are producers of proteases and are present in increased numbers in COPD. Matrix metalloproteinases (MMPs) are a group of over 20 closely related endopeptidases produced by both neutrophils and alveolar macrophages as well as airway epithelial cells (224). Activities of collagenase (MMPs-1 & 8) and gelatinase B (MMP-9) are elevated in BAL fluid in emphysema in comparison to healthy controls (225;226). Whilst neutrophil elastase and gelatinase B activities may be increased as a direct result of smoking, increased collagenase activity seems to be more specific for COPD (227). Alveolar macrophages cultured from BAL fluid express increased levels of mRNA transcripts for MMP-9 in emphysema, but no differences are seen between disease and health for MMP-1 or MMP-12 mRNA transcript levels (228). Increased MMP-9 levels and activity have also been demonstrated in induced sputum in COPD (229). Increased MMP-1 levels and activity have also been demonstrated in emphysematous lung parenchyma, suggesting that MMP-1 may play a key role in the pathogenesis of emphysema.

1.2.3.6 Receptors, kinases and transcription factors

The cytokines and chemokines discussed in section 1.2.3.4 act through a series of receptors. IL-8 binds to both CXCR1 and CXCR2. CXCR2 is also activated by other mediators relevant to the development of COPD (230). CD8⁺ cells show increased expression on CXCR3 in the airways of patients with COPD. (231) Mitogen-activated protein (MAP) kinases are associated with chronic inflammation and the p38 MAP kinase pathway is activated by cellular stress
and regulates the production of inflammatory cytokines, including IL-8, TNF-α and MMPs. (232;233). In a murine macrophage model of oxidant stress generated by ischaemia leads to activation of p38 MAPK is an Src-dependent manner and P38 does not have a direct role in inducing oxidant dependent NF-κB translocation (234).

The transcription factor NF-κB regulates the expression of IL-8, TNF-α and some matrix metalloproteinases. Acute exposure to cigarette smoke causes expression of NF-κB through a superoxide dependent mechanism, which results in neutrophil accumulation in the airways (235). NF-κB has been shown to be activated in the macrophages and epithelial cells of COPD patients and this is particularly pronounced during exacerbations (236;237). It has been shown that smoke induced activation of human monocytes and macrophages to produce IL-8 is oxidant mediated via the activator protein-1 pathway, but not the NF-κB pathway (238). Toll-like receptors (TLRs) are essential for the immune response to invading micro-organisms, and they may have a significant role to play in the development of the chronic inflammation that is seen in COPD (239). TLRs are a pattern recognition receptor (PRR) and are part of the innate immune system and bind to molecules that are shared by pathogens but are distinct from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). Thirteen types of TLRs have been discovered in humans, and activation triggers a series of signalling pathways that results in the activation of transcription factors such as NF-κB (240). The TLRs of particular interest in COPD are TLR2 and TLR4 as they have been shown to be involved in the response to cigarette smoke challenge, LPS and oxidant stress (241;242)
Figure 1.4 shows the inflammatory changes that occur in COPD as a result of cigarette smoke exposure.

1.2.3.7 Nasal disease in COPD

Epidemiological studies have shown that rhinosinusitis occurs in COPD and may be associated with significant nasal symptoms (112;112). The severity of nasal obstruction is proportional to the severity of airway obstruction and hence disease severity (243). Nasal inflammation is also seen to increase in parallel to bronchial inflammation at the time of exacerbations of COPD (244). There is also a direct link between the development of nasal symptoms, including rhinorrhea, nasal congestion and sneezing, and cigarette smoking (245;246). Passive exposure to cigarette smoke also causes inflammation of the nasal mucosa (247), and nasal obstruction as measured by rhinomanometry (248). The mechanism of the nasal inflammation is not clear, although it is likely to be similar to that found in the lungs. Patients with COPD have increased numbers of neutrophils in both nasal and bronchial biopsy specimens (249). It has also
been shown that patients with COPD have increased nasal neutrophil activity and increased mucinous secretory responsiveness (250). Some of the effects of smoking may also be due to the deleterious effect of cigarette smoke on mucociliary clearance (251).

### 1.3 Current treatment and potential new therapeutic agents for allergic rhinitis, asthma and chronic obstructive pulmonary disease

#### 1.3.1 Allergic rhinitis

Current pharmacological treatment of allergic rhinitis involves anti-histamines, steroids and immunotherapy. Oral H1 antihistamines are taken daily and are rapidly effective on some nasal and eye symptoms but are only moderately effective on nasal obstruction. Intranasal glucocorticoids are the most effective therapy for allergic rhinitis and are effective on nasal congestion. The maximal effect is seen after a few days of use, and their use is recommended in the weeks preceding the pollen season. If symptoms are particularly severe then a short course of oral glucocorticoids may be necessary.

Allergen avoidance is a part of the management of allergic disease. Severity of asthma is related to the level of allergen exposure (252;253). However the relationship between levels of exposure and asthma symptoms is complex, with differing levels of allergen required to trigger symptoms (254). Atopic asthmatics who are exposed to high levels of house dust mite or dog allergens but are not sensitized to these allergens as determined by skin prick testing have increased airway reactivity (255).
There are significant difficulties in obtaining a randomised blinded trial removing bias in assessing allergen avoidance and several studies have provided conflicting evidence on the benefits of removing allergens from the home (256). Removing the allergen can be difficult, with domestic pets it has been shown that removal of the cat from living room and bedroom areas and the use of a HEPA (high efficiency particulate air) filtration can reduce airborne levels of Fel d1 in homes.(257). A randomized trial of the use of air filters and HEPA vacuum cleaners in 30 sensitized asthmatics who shared a home with cats or dogs resulted in a small but significant improvement in bronchial hyper-reactivity or a reduction in treatment by one step on the British Thoracic Society guidelines. There were however no changes in lung function or levels of airborne pet allergen (258).

The removal of house dust mite allergen is a lot more problematic. The use of vacuum cleaners can actually lead to an increase in allergen exposure, and HEPA cleaners do not reduce the level of allergen exposure. The use of special bed lines has been proposed to reduce exposure to house dust mite allergen, however this intervention has not been shown to bring about symptom control in adults, but children showed an improvement in asthma control(259). A large study of the use of allergen-impermeable bed covers in asthmatic patients has shown no clinical benefit as determined by the success of a steroid weaning programme (260).

Allergen avoidance should bring about significant improvement in seasonal allergic rhinitis as patients do not suffer any symptoms outside of the pollen season. However avoidance of pollen can be difficult but there is evidence that nasal filters reduce both the nasal and ocular symptoms associated with pollen
exposure (261). The many trials of allergen avoidance are reflected in the recent British Society for Allergy and Clinical Immunology guidelines suggest advising allergen avoidance for allergy to domestic pets, horses and occupational allergens such as latex (262).

Leukotriene receptor antagonists have a role to play in the treatment of seasonal allergic rhinitis (263), although not all patients show benefit (264). A recent systematic review and meta-analysis of the use of montelukast in allergic rhinitis has concluded that montelukast does reduce nasal symptom scores, but is not as effective as topical nasal steroids and when used it should be in combination with an anti-histamine (265).

Nasal douching with saline is simple procedure that can be used as an adjunct to pharmacological therapies in allergic rhinitis (266;267). Topical cromones have an undefined mechanism of action, and are particularly effective for eye symptoms however their intranasal use is less effective. Sodium chromoglycate only has very limited activity in the treatment of allergic rhinitis (268).

Oral decongestants are sympathomimetic drugs that act to reduce the symptoms of nasal congestion. They are often poorly tolerated as they have a poor side effect profile and have no effect on the other symptoms of rhinitis.

1.3.2 Asthma

The aim of asthma treatment is to minimise asthma symptoms and to limit its effects on activities of daily living. Avoiding triggers is important, especially in atopic asthma. A stepwise approach to the treatment of asthma has been recommended by the Global Initiative for Asthma (GINA) work group (7;269). The latest issue of the guidelines emphasise control of asthma symptoms. The following table summarises the advice given in the GINA guidelines. Patients
should be reviewed regularly, and if stable for a three month period then treatment should be stepped down.

A proportion of patients will continue to have persistent symptoms despite being on maximal therapy as defined by GINA. Attempts have been made to reduce the dose of oral corticosteroids by using other anti-inflammatory therapies such as methotrexate and cyclosporine. There is limited evidence that these therapies achieve that goal but they are associated with a significant side effect profile (270;271). Specific targeting of the CD4 T cell population using a monoclonal antibody has also been trialled in the treatment of severe corticosteroid dependent asthma (272).

A major change in the way asthma medication is prescribed has been achieved by a regimen known as SMART (Symbicort Maintenance And Relief Therapy). Symbicort is a combined inhaler consisting of an Inhaled Corticosteroid (ICS) and Long-Acting β2-Agonist (LABA) that has a licence to be taken as a regular maintenance treatment and as needed in response to symptoms. There have been 8 published large RCTs that have assessed the impact of SMART compared with fixed doses of ICS and showed a reduction in severe exacerbations and a lower total ICS dose (273-280).
<table>
<thead>
<tr>
<th>Level of severity</th>
<th>Daily Controller Medications</th>
<th>Other treatment options</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEP 1 Intermittent</td>
<td>None necessary</td>
<td>As need bronchodilator</td>
</tr>
<tr>
<td>STEP 2 Mild persistent</td>
<td>Low dose Inhaled CorticoSteroid (ICS)</td>
<td>Sustained release theophylline or Cromone or Leukotriene modifier</td>
</tr>
<tr>
<td>STEP 3 Moderate persistent</td>
<td>Low to medium dose ICS plus long acting $\beta_2$ -agonist (LABA)</td>
<td>Medium dose ICS plus sustained release theophylline OR Medium dose ICS plus LABA OR High dose ICS OR Medium dose ICS plus leukotriene modifier</td>
</tr>
<tr>
<td>STEP 4 Severe persistent</td>
<td>High dose ICS plus LABA one or more of the following Sustained release theophylline Leukotriene modifier Long acting oral $\beta_2$-agonist Oral corticosteroid</td>
<td></td>
</tr>
</tbody>
</table>

1.3.3 New treatments for allergy and asthma

There is a need for new treatments as although corticosteroids are very effective, they are associated with side effects which limit compliance, and there is a group of patients with severe asthma who do not respond to treatment with corticosteroids (281).

There have been two main new therapies in the treatment of allergic disease in recent years. A humanised anti-IgE monoclonal antibody (Xolair, Novartis-Genentech) has a licence for the treatment of severe persistent asthma not responding to high dose inhaled corticosteroids and long acting beta agonists (282). A novel immunotherapy agent using sublingual immunotherapy (SLIT) involving grass pollen is now available, and hopefully will improve access to this type of therapy that was previously given by subcutaneous injection (283).
Previous attempts at directed anti-inflammatory therapies have been disappointing. Soluble IL-4 receptor (Nuvance, Immunex) failed to show clinical efficacy in large scale trials after promising results in early phase trials (284;285). An anti-IL-5 antibody has been shown to reduce blood eosinophils but does not remove eosinophils from airway walls (286) and has been shown to have limited efficacy in moderate (287) and severe asthma (58). However anti-IL-5 has been shown to reduce eosinophil numbers and tenascin deposition in allergen challenged human atopic skin (59), as well as extracellular matrix in bronchial subepithelial basement membrane (59). Although disappointing in asthma and eczema anti-IL5 has been shown to be effective in hypereosinophilic syndrome with skin manifestations (288-290), eosinophilic oesophagitis (291). Anti-IL5 has also been shown to reduce the size of nasal polyps in 50% of patients (292). A soluble TNFa receptor construct has been shown to be effective in a small group of patients with severe asthma (n=10) (293), while an antibody directed against TNF-α (Remicade, Centocor) has been shown to decrease exacerbation rate in moderate asthma (294).

Dendritic cells have a role to play in asthma as they present antigen to Th2 cells and their activity is stimulated by thymic stromal lymphopoietin (TSLP) (295). Hence blocking the effects of TSLP may have significant effects on the development of allergic disease. Regulatory T cells also have a role in modifying the immune response, and it has been shown that immunotherapy increases IL-10 production by regulatory T cells (296). Hence therapies that enhance T regulatory cells function and increase IL-10 production are being developed. IL-12 acts to suppress Th2 cells, and administration of IL-12 reduces levels of blood eosinophils, but does not reduce responsiveness to inhaled allergen and has a poor side effect profile (297). Administration of T cell
peptides has the potential to achieve the same effects as immunotherapy but with an improved safety profile (298).

Targeting cytokines is also likely to yield new agents to target allergic disease, although as discussed above clinical trials have so far been disappointing. IL-9, IL-15, IL-17, IL-18 and IL-21 are all potential targets to modulate responses in allergic disease. All of the mediators of allergic disease act through receptors which in turn trigger intra-cellular signalling. The commonest signalling pathways in allergic disease are NF-κB and P38 MAP kinase and inhibitors of these are available (299;300). A major limitation is that these enzymes are ubiquitous in human cells and hence may need to be given by inhalation to prevent unacceptable side effects. At a protein synthesis level, antisense-oligodeoxynucleotides and interference RNA (RNAi) can inhibit the production of inflammatory mediators (301;302).

1.3.4 COPD

The most important therapeutic intervention is smoking cessation and patients should be given enough encouragement and pharmacological support to achieve this. Vaccination against influenza and pneumococcus also play an important preventive role in the treatment of COPD. The GOLD guidelines provide a stepwise approach to treatment based on lung function determined severity (303). Mild COPD with an FEV$_1$ of $\geq$ 80% predicted can be treated with an inhaled short acting bronchodilator when needed. For moderate COPD with an FEV$_1$ $<$ 80% predicted but $\geq$ 50% predicted a long acting bronchodilator should be added and the patient considered for a pulmonary rehabilitation program. For severe COPD with an FEV$_1$ $<$ 50% predicted but $\geq$ 30% predicted the management should be as for moderate COPD but inhaled corticosteroids
(ICS) should be added if there are frequent exacerbations (304). Very severe COPD is defined as and FEV\textsubscript{1} < 30% predicted or < 50% predicted with chronic respiratory failure, and should be managed as for severe COPD with the addition of long term oxygen therapy if there is chronic respiratory failure and surgical treatments including lung transplantation should be considered. Bronchodilators have been shown to improve exercise capacity independently of their effects on lung function (305;306), and combinations of bronchodilators with differing mechanisms of action leads to a synergistic effect (307;308).

Development of new therapies to aid in smoking cessation or improved bronchodilators and inhaled corticosteroids are all areas of great interest to pharmaceutical companies working towards improvements in the management of COPD.

As the pathophysiology of COPD becomes more defined and the precise cellular pathways involved in inflammation then potential new targets for drugs can be identified. There is also considerable evidence that oxidative stress plays a large role in the development of COPD (146), and it has been suggested that targeting this would bring benefits (309). However studies with oral N-Acetyl Cysteine (NAC) have shown a small but significant reduction in exacerbation frequency (310), and more effective antioxidants may bring about greater clinical effects. Selective inhibitors of iNOS have been developed and these may result in reduced production of free radicals in the airways (311).

Phosphodiesterase (PDE4) is the predominant PDE in neutrophils, CD8 T cells and macrophages (312) and hence is a logical choice to target to reduce inflammation in COPD. The PDE4 inhibitor Cilomilast (GSK) has been shown to reduce CD8\textsuperscript{+} and CD68\textsuperscript{+} cells in the airways of patients with COPD, however
this did not result in any changes to sputum parameters or FEV₁ (313). However in a larger study Cilomilast did cause a reduction in trough FEV₁, although the reduction was small and quality of life outcome measures were unchanged (314). The main drawback with the PDE4 inhibitors is that the dose is limited by the gastro-intestinal side effects of nausea and loss of appetite.

Leukotriene B₄ is a potent chemoattractant for neutrophils and is increased in the sputum of patients with COPD (204) and a receptor antagonist has been shown to inhibit the neutrophil chemotactic activity of COPD sputum (315). The CXC chemokines IL-8 and GRO-α are increased in COPD and act via the CXCR2 receptor, hence antagonists of this are potential new therapies. The CC chemokine MCP-1 is also implicated in the pathogenesis of COPD suggesting that CCR2 receptor antagonists may also be useful. TNF-α is also raised in COPD and early phase trials of a TNF-α antibody in asthma have been promising (294). However a study of the use of a monoclonal antibody against TNF-α (Remicade, Centocor) did not show any benefit in moderate to severe COPD and there were increased rates of malignancy in the treatment group (316).

The protease, anti-proteinase balance is also a potential site for drug development either through delivery of inhibitors of proteases or administration of anti-proteases such as α₁-antitrypsin of secretory leukoproteinase activity. The recruitment of inflammatory cells into the airways is dependent on the presence of adhesion molecules on the surface of the cells and hence inhibitors of these molecules would seem to be a good target for an anti-inflammatory treatment. However these adhesion molecules also form an essential part of the defence mechanism against invading pathogens and hence may be associated
with an unacceptable level of immunosuppression. p38 MAP kinase is involved in the intra-cellular cascade that leads to the expression of inflammatory cytokines and chemokines and inhibitors of these have been developed and may have useful anti-inflammatory effects (317). Attempts at regeneration of lung tissue using retinoic acid has been disappointing (318), but stem cell regeneration has the potential to be used in lung regeneration (319).

1.4 Non-invasive assessment (biomarkers), challenge models, and early clinical trials of novel anti-inflammatory drugs

A biomarker is a molecular indicator of a specific biological property that can be detected ex vivo and can be used to measure the progress of disease or the effects of treatment. Biomarkers can be used as an indicator of normal biological processes, pathogenic processes, and pharmacologic responses. Biomarkers are surrogate markers that may correlate with a clinical endpoint (320). It has been argued that waiting for clinical endpoints to be measured can lead to long delays in the introduction of new drugs and the use of a surrogate biomarker can expedite the introduction of a new drug (321). Due to the burden of respiratory disease, there is a need for new therapeutic agents. Based on the numerous potential new anti-inflammatory drugs, there is the need for models to allow rapid assessment of new molecular entities. Translational medicine is a rapidly expanding field and aims to speed the process between new scientific insights, drug target identification, and clinical trials to assess novel treatments, that permit new drugs to become licensed for use in patients.

To improve safety of phase 1 trials it is vital to have in vitro methods of assessing effects in human models. Because of species differences it is not always possible to transfer safety data from animal studies to humans. This is
particularly relevant in the development of human monoclonal antibodies the
effects of which cannot be reliably carried across species. The catastrophic
events that occurred during the testing of the monoclonal antibody (MoAb)
against CD28, TGN 1412 in March 2006 at The Parexel research unit located at
Northwick Park Hospital only re-enforce the need to be sure of a drugs full
range of effects before administration to volunteers. The drug was given by
rapid IV infusion to 6 previously healthy volunteers, and they went on to develop
multi-organ failure due to a cytokine storm (322). At high doses TGN 1412
becomes a superagonist causing T cell cytokine release, and we require human
blood in vitro safety tests to identify potential toxic effects of biological therapies
such as TGN 1412 (323) To aid assessment of new therapies, it would be
useful if a model could be developed that allowed effect to be determined after
a single dose of some drugs. This would also have the benefit of limiting patient
exposure to drug. If a compound showed limited effects in these early studies
then it would not be worth pursuing in larger scale trials. It is important however
to note that efficacy in the pre-clinical models does not guarantee clinical effects
in the target patient group.

Animal allergic models are generally based on murine ovalbumin sensitisation
which allows small amounts of new molecular entities to be assessed for anti-
eosinophilic effects. The study of seasonal allergic rhinitis is affected by the
short pollen season which make cross-over studies impossible to carry out, and
the pollen exposure can vary daily hence relying on the naturally occurring
disease state can be difficult. It is advantageous to have a model that
challenges with a quantified allergen dose out of the pollen season to allow for
cross over design trials. To mimic allergen exposure it is possible to utilise a
Vienna chamber, which is a closed unit where allergen is introduced and the
subjects are exposed in a way that mimics their normal allergen exposure. This has the advantage of allowing a large number of subjects to be exposed at once. It is also possible to directly apply allergen to the mucosa via a nasal spray; this is a quick and reliable way to apply allergen specifically to the area of interest. An alternative human model is intradermal administration of allergen which has proved to be a useful model of immune response to allergen (324;325).

To mimic the allergic responses in asthma the inhaled allergen challenge is used, where subjects inhale an antigen they are allergic to and then undergo airway sampling either by inducing sputum or obtaining samples bronchoscopically. However this can sometimes provoke dramatic fall in lung function, and repeated challenge and airway sampling at frequent intervals is not possible. Alternative models of asthma have been developed such as inhaled methacholine, which is a non-selective muscarinic receptor agonist acting on bronchial smooth muscle.. There was a death in a healthy volunteer who inhaled hexamethonium, an acetylcholine receptor antagonist, as part of a study investigating the inflammatory responses in asthma. Careful review following this death concluded that there had been some data presented in the 1950's that had indicated a problem with this challenge model and one paper failed to include details of problems in subjects because breathlessness and cough had been attributed to viral infections (326).

Challenge models for COPD are either currently animal models such as the smoking mouse model or in vitro human models such as cigarette smoke conditioned medium or LPS challenge of isolated human lung macrophages. There is currently no safe, reliable in vivo human model of COPD. It has been
suggested that inhaled LPS would be a good model for testing new drugs in COPD, however very careful consideration must be given to the safety of this. It is important to remember that patients with COPD are considerably older than subjects undergoing allergen challenge, and as a consequence of this and their smoking history often have co-existing disease. Therefore any challenge models in this group of patients must be the least invasive possible and ideally avoid the need for any procedures which may cause a reduction in lung function.

It can be seen that it would be extremely useful to have human blood and nasal challenge models to assess new therapies for allergic disease and COPD. For allergic disease it is relatively easy to mimic the acute and late phase reactions by administering allergen. However the situation with COPD is much more complex as the disease arises from chronic exposure to a stimulus, most commonly cigarette smoke, over a long period of time hence developing an acute model is much more difficult. The stimulus is also much more complex, allergen can be extracted and purified, but the components of cigarette smoke are too numerous to study individually. So in this Thesis the stimulus was either cigarette smoke or systems which generate oxidative stress to reproduce the oxidative stress component of COPD.

The nasal mucosa is very similar to the respiratory mucosa lining the lungs and is in continuation with it. This means that the nasal mucosa could potentially be utilised as a surrogate for inhaled challenge models. The main differences are that the nasal sub-epithelium has a large network of blood vessels and is devoid of smooth muscle. Table 1.3 shows the advantages of the nasal challenge model over inhaled challenge models.
Table 1.3 Comparison of single inhaled and nasal allergen challenge models

<table>
<thead>
<tr>
<th>Inhaled</th>
<th>Nasal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Requires inhalation from a nebuliser</td>
<td>Single nasal spray</td>
</tr>
<tr>
<td>Single inhaled allergen challenge is generally repeated at 3 weekly intervals</td>
<td>Single NAC is generally repeated at 2 weekly intervals. Can be given daily</td>
</tr>
<tr>
<td>Limited number of airway samples can be collected, e.g. sputum at 24h</td>
<td>Nasal samples can be taken every 5 minutes</td>
</tr>
<tr>
<td>Potential for decline in lung function, safety concerns over anaphylaxis and decreased lung function are greater</td>
<td>Minimal potential for allergen to reach the lungs</td>
</tr>
<tr>
<td>Requirement for allergic asthmatics off inhaled corticosteroids (ICS) can lead to study recruitment difficulties.</td>
<td>Patients with hayfever out of season can be readily recruited</td>
</tr>
</tbody>
</table>

Whole blood stimulation assays can be used early in drug development to determine pharmacological activity of new drugs. This can be done purely as an in vitro assay whereby drug is added to whole blood and responses measured, or as an ex vivo model whereby subjects are given the drug and responsiveness to whole blood challenge measured at time intervals following drug ingestion. A combination of these methods allows assessment of drug efficacy and provides information about pharmacodynamic properties.

1.5 Aims

Asthma and COPD are common illnesses seen in both primary and secondary care. Currently the main treatment is bronchodilators and inhaled steroids. There is considerable evidence that there is ongoing inflammation in both of these conditions and novel therapeutic agents could be developed to target this inflammation. The development of new drugs requires robust models for testing their efficacy. Conventionally new drugs for respiratory disease have been tested using inhaled challenge models, which are invasive and have limited...
opportunities to obtain samples following challenge. This thesis develops a
nasal challenge model for testing novel therapeutic agents for allergic disease
and nasal and blood challenge models of COPD.
2  GENERAL METHODS

2.1 Clinical

2.1.1 Subject selection

Subjects with allergic rhinitis were generally recruited from newspaper advertisements. Subjects with COPD were recruited from outpatient clinics at The Royal Brompton Hospital and St Mary's Hospital, Paddington. Subjects were also recruited from local smoking cessation courses and through advertisements placed in local newspapers. All studies received ethical approval and all subjects gave informed consent.

2.1.2 Medical and smoking history

All patients had a full medical history taken by a research physician on the Clinical Studies Unit, with particular note taken of previous respiratory illness and any recent viral illnesses. Patients were carefully questioned regarding any drugs taken including any over the counter preparations. Details of smoking habits were taken and a calculation of "pack years" made.

2.1.3 Lung function

Spirometry was performed using a computerised dry bellows Vitalograph spirometer which was calibrated at the start of each day. The patient was asked to take a full inspiration and form a tight seal around the mouthpiece and to perform a full forced expiration. The best of three attempts was used. Comparison was made to the predicted values from the European Coal and Steel workers (327).
2.1.4 Skin prick testing

Intra-epidermal challenge with an allergen extract was used to determine sensitivity to Timothy grass extract. The tests were performed on the volar aspect of the forearm. Test sites were marked, and a single drop of negative control, positive control and Timothy grass solution was placed on the skin. A sterile lancet was introduced into the epidermis through the solution, and the remaining solution blotted off with a tissue. After 15 minutes the wheal size was measured using a transparent ruler. A positive result was taken to be a wheal ≥3mm more than the negative control.

2.1.5 Nasal symptom scores

Subjects completed symptom score cards supplied during the study and graded their symptoms as detailed. Subjects were not allowed to ask for guidance from the clinical staff when completing the forms. An example of a symptom score card is included in the appendix. A grading from 0-3 was given to the four symptoms of nasal blockage, rhinorrhea, sneezing and nasal itch giving a maximum possible score of 12. This was recorded prior to nasal challenge and at time points following challenge as detailed in the study protocol.

2.1.6 Nasal lavage collection

Nasal lavage was performed using a modified nasal pool technique (328). Nasal lavage was performed using a nasal olive attached to a 10ml syringe containing 5mls of PBS at room temperature. A nasal olive of appropriate size was placed gently into the anterior nares to make a close fitting seal to prevent leakage of lavage fluid. The subject was seated with the neck flexed to 50° from the upright position to prevent fluid reaching the nasopharynx. The PBS was slowly instilled in to the left nasal cavity and allowed to remain there for several seconds. This
was repeated 20 times to maximise collection for cell phase data. The volume of PBS recovered after lavage was noted and the sample placed on ice and transported to the laboratory where it was processed immediately. To ensure comparable baseline nasal lavage, an initial lavage was carried out in both nostrils prior to collection of baseline lavage and filter paper samples and the lavage fluid recovered discarded.

2.1.7 Nasal filter paper technique

A modified "matrix method" was used adapted from Alam and Sim (95;329;330). Two strips (7x 30 mm) of filter paper with rounded edges (Whatman No. 40; Whatman paper Ltd, Maidstone UK) were placed onto the lateral wall of the right nasal cavity and left for 2 minutes. After removal from the nose the strips were placed into a labelled eppendorf® containing 500μL of Luminex® assay buffer. This was then placed on ice and transferred immediately to the laboratory for processing.

2.1.8 Blood collection for flow cytometry studies

Blood was taken from an antecubital arm vein using a tourniquet. Blood was slowly withdrawn into a syringe using a 0.6mm internal diameter butterfly needle. (Venisystems). The blood was then placed immediately into a collection tube containing EDTA. A 0.25M solution of EDTA was used. 100μL was added for each 3mls of blood collected for incubations up to 30 minutes. For more prolonged incubations 200μL EDTA was added for each 3mls of blood collected. In experiments using washed cells 3mls whole blood was made up to 12mls using PBS and spun at 400g for 10 minutes. The supernatant was removed taking care not to disturb the buffy coat; the cell suspension was then made up to 3mls using PBS.
2.1.9 Blood collection for cytokine/chemokine analysis

An 18G cannula was inserted into an ante-cubital vein and flushed with 2.5mls normal saline to ensure patency. 5mls of blood was withdrawn and discarded prior to study blood sampling. 4.5mls blood was withdrawn using a vacuum collection system into a tube containing powdered EDTA and placed immediately on ice. This was then spun at 1000g at 4°C for 10 minutes and the plasma removed taking care not to disturb the buffy coat. The plasma was aliquoted into labelled cryo tubes. 5ml blood was collected into a SST tube and left to stand for 30 minutes at room temperature. This sample was then spun at 1000g for 20 minutes at room temperature and the serum carefully removed and aliquoted into labelled cryo tubes. The plasma and serum samples were stored at -20°C until analysis.

2.2 Laboratory

2.2.1 Nasal Lavage processing

The total volume of nasal lavage recovered was centrifuged at 4°C for 10 minutes at 400g. The supernatant was then aliquoted using a 3ml disposable wide bore pipette into a 15 ml polypropylene centrifuge tube and this was mixed using a vortex to ensure that the sample was homogenous. Three 500μL aliquots were transferred to labelled cryo tubes; these were stored at −80°C for latter analysis. The remaining cell pellet was resuspended in 5mls of 0.1% DTT prepared fresh daily from a 1% stock. This cell suspension was gently aspirated with a 3 ml disposable wide bore plastic pipette. The sample was gently agitated on a rolling mixer for 10 minutes. The nasal lavage was again centrifuged at 4°C for 10 minutes at 400g. The DTT containing supernatant was removed using a 3ml disposable wide bore pipette and discarded. The
remaining pellet was resuspended in PBS to make a total volume of 210 µl. 10 µl of sputum cell suspension was added to 10 µl of trypan blue (1 in 2 dilution). 10 µl of the mixture was placed under the microscope for cell counting using an improved Neubauer haemocytometer. If the cells were too dense for an accurate count, the procedure was repeated with additional dilution until satisfactory for an accurate count. The dilution was noted. Cells are counted in the bottom left, middle and top right quadrants of the haemocytometer grid. The total leukocyte count per ml is calculated, taking into account the dilution factor of trypan blue, by using the following formula:

\[
\text{Total leukocyte count (cells/ml)} = (\text{Average no of viable + dead leukocytes}) \times \text{trypan blue dilution factor} \times 2000
\]

One cytospin block was prepared, using the remaining 200 µl resuspended cell pellet. The cytospin slides are produced by spinning at 450 rpm, medium acceleration, for 3 minutes. The slides were left to air-dry for 30 min, and then fixed in methanol. The slides were stained with Diff-Quik® stain, mounted using size 0 cover slip and DPX, and stored at room temperature. A BSA solution was added to the lavage supernatant to give a final 2% concentration, the lavage supernatants were then centrifuged through a 0.22 µm pore size cellulose acetate filters (Costar spin-X®) at 4°C for 30 min at 2000G to remove residual mucus before analysis.

Nasal lavage samples were not corrected for variations in recovery rate, as it has been shown that levels of eotaxin recovered in nasal lavage fluid was unchanged when corrected for albumin concentration in lavage fluid (98).
2.2.2 Nasal filter paper processing

The papers in assay buffer were mixed using a vortex for 15 seconds and then placed on the rolling mixer for 10 minutes to allow mediators to elute. After centrifugation at 4°C for 10 minutes at 400g, the eluate was collected and stored at -70°C for later analysis. A BSA solution was added to the eluate to give a final concentration of 2% and the eluate was then centrifuged through a 0.22µm pore size cellulose acetate filter (Costar spin-X ®) at 4°C for 30 min at 2000G to remove residual mucus before analysis.

2.2.3 Quantification of cytokine and chemokine levels using Luminex® multiplex analysis

Cytokine and chemokine analysis of nasal and sputum samples was carried out on the Luminex100®IS™ analyser (Luminex Corporation, Austin, Texas) Multiplexed bead array systems were used (Upstate Biotechnology, Milton Keynes, UK). A standard curve in duplicate was used for each sample run. Data was analysed with either Luminex or STARstation software.

The Luminex bead array system enables multiple cytokines and chemokines to be analysed on a single 50µL sample. The system works by coupling a capture antibody against the cytokine to be tested to one of a hundred uniquely colour coded polystyrene microspheres. Then a detection antibody labelled with the fluorochrome phycoerythrin is added and the sample passed through a flow cell and exposed to a laser with surrounding detectors. The quantification of antigen-antibody reactions that occur on the microsphere surface can be performed by measurement of the relative fluorescence intensity. This can then be read against a standard curve to give concentrations of the cytokine under study(331). It is the presence of the uniquely coloured microspheres that allows
for multiple cytokines to be measured. Comparative experiments have shown that cytokine multiplex assays are comparable in sensitivity, accuracy and reproducibility to Enzyme Linked Immunosorbant Assays (ELISAs) (332) (333). It has been proposed that multiplexed assays could be used as a screening tool that could be used to identify a promising marker that could then be analysed in future experiments using a specific ELISA (334).

2.2.4 Statistical analysis

All graphs and statistical analyses were generated by using the GraphPad Prism® 4 graphing and statistical package.
3 Single dose nasal allergen challenge: repeatability and
dose response of nasal allergen challenge on symptom
scores, eosinophils and inflammatory mediators

3.1 Abstract

Introduction: Nasal allergen challenge (NAC) is a simple method for the investigation of the inflammatory response that occurs following allergen challenge. Multiple samples can be obtained following a single challenge. The challenge can be repeated after a 14 day washout period to determine repeatability and dose response effects.

Aims: This study aims to determine the dose response and degree of repeatability of nasal allergen challenge with regard to detectable levels of inflammatory mediators and to enable power calculations for future studies with new therapies.

Methods: Patients with grass pollen seasonal allergic rhinitis (n=8) outside the pollen season were given topical NAC with 500 BU of Timothy grass pollen on 2 consecutive visits with a two week washout period. A separate cohort of patients (n=8) were challenged with 50BU, 250BU and 500 BU in a randomly assigned order to assess dose response.

Results: All subjects had symptomatic nasal allergic responses following NAC. In nasal filter paper eluate there were not consistent significant changes in levels of interleukin (IL)-4, IL-5 and IL-13, and there were generally high and variable background levels compared with a previous published study. In a similar manner, nasal lavage did not contain consistently elevated levels of IL-4, IL-5 and IL-13 in the late phase. Elevations in lavages levels from baseline in
the first 60 min may have been due to a falsely low baseline immediately after washing, followed by a leaking back of cytokines into the nasal lining fluid. In contrast, nasal C3a and PGD2 were elevated in nasal paper eluates during the early phase, however they were poorly repeatable. There was a dose response for C3a in the early phase.

**Conclusion:** The main endpoint in this study was to determine levels of IL-4, IL-5 and IL-13 in the late phase, and there was failure to detect consistent late phase responses in both filter paper eluate and nasal lavage. The reasons for this disappointing result were thought to be methodological and the subject of a detailed review. In a later study steps were taken to increase the allergen dose (x3) and employ a new type of synthetic absorptive matrix (SAM) instead of Whatman's filter paper (See Appendix 2).

### 3.2 Introduction

#### 3.2.1 Use of nasal allergen challenge

Inhaled allergen challenge has been used in clinical studies to study the inflammatory response in the allergic asthmatic reaction and to assess the response to treatment (335). The respiratory tract in the nose shares many features with the lung as part of the “one airway hypothesis” (336;337). The use of a nasal challenge model has several advantages over inhaled allergen challenge including ease of recruitment, subject safety and the ability to obtain samples at multiple time points. This ability to obtain repeated samples allows both the early and late phase levels of mediators to be determined. Symptom scores can be collected following NAC using a questionnaire completed by subjects. The nasal mucosa can be lavaged with physiological saline to obtain fluid for analysis (328). This nasal lavage yields both a fluid phase for mediator
analysis and also a cell phase for determination of eosinophil influx. Mediators can also be detected in nasal secretions using a modified matrix filter paper method (329). This has the advantage over nasal lavage samples given that dilution of the mediators does not occur. Allergen challenge of the nasal epithelium could be used to test new therapeutic agents in the treatment of allergic rhinitis and potentially also new anti-inflammatory therapies for asthma due to their similar pathogenesis.

3.2.2 Nasal responses following allergen challenge

Symptom scores have been studied following nasal allergen challenge (NAC). There is a well defined early phase of sneezing, rhinorrhoea and blockage. In contrast to inhaled allergen challenge there is not a well defined late phase symptom response (338). Allergen challenge of allergic individuals has been shown to produce nasal symptoms and increase levels of histamine and tryptase in nasal lavage fluid, and these changes are not seen in non-allergic subjects (44). Topical corticosteroids have been shown to reduce symptom scores in relation to allergen challenge (100;101).

Prostaglandin D$_2$ (PGD$_2$) is produced by mast cells and has been detected in high concentrations at the site of allergic inflammation (46;47). It has been shown that PGD$_2$ stimulates the production of IL-4, IL-5 and IL-13 by human Th2 cells at levels found at the site of allergic inflammation, and gene transcription peaks at 2 hours (77). $\beta$-Tryptase is a serine protease that is released from degranulating mast cells and has inflammatory actions through PAR-2 activation (48). In addition tryptase may also contribute to airway oedema through microvascular leakage (49). In a NAC study a reversible $\beta$-
tryptase and trypsin inhibitor has been shown to reduce symptoms, eosinophils, and levels of IL-5 following NAC (50).

The production of TH2-type cytokines such as IL-4 and IL-5 are characteristic of allergic diseases. A study of nasal biopsies at 6 hours following nasal allergen challenge has shown that the levels of eosinophils (but not T-cells) increase in nasal mucosa. Co-localisation studies showed that the majority of the IL-4 and IL-5 mRNA was associated with eosinophils (94). This is important because it suggests that eosinophils are the source of early phase cytokines that lead to the development of a late phase response. IL-5 levels have also been shown to increase in nasal filter paper samples during the early and late phase following nasal allergen challenge; however IL-4 was not detected in this study (95). Another biopsy study has shown that NAC induces a significant tissue eosinophilia with an associated increase in IL-8, IL-13 and RANTES mRNA-positive cells and that increased levels of IL-13 mRNA-positive cells are still present 1 week following single NAC (96). A co-localisation study has determined that the majority of RANTES mRNA-positive cells following NAC are macrophages, followed by eosinophils (97).

Following NAC there are parallel increases in eosinophil counts, eosinophil protein X, and eotaxin concentrations in nasal lavage fluid (98). Nasal allergen challenge has been shown to increase levels of the cytokines IL-4, IL-5 and IL-13 in filter paper samples collected during the late phase. The chemokines eotaxin, RANTES, MCP-1, MIP-1α, IL-8 and IP-10 were also produced during the late phase. Pre-treatment for a week with topical fluticasone, a corticosteroid inhibited this response to nasal allergen challenge. Fluticasone also reduced symptom scores and eosinophil influx into nasal lavage samples
following nasal allergen challenge (100). Using an anti-IL-5 antibody, it has been shown in a guinea pig model of allergic rhinitis that IL-5 is essential for eosinophil migration from the bone marrow to the nasal airway. However neither IL-5 nor eosinophils are required for the development of nasal symptoms and nasal hyperresponsiveness in this animal model (99). Interestingly levels of TH1 cytokines (IL-2, IL-3, IL-7, IL-12, IL-15, TNF-α, IFN-γ, GM-CSF) were not. It has also been shown that a single dose of nasal steroid has the ability to abolish the IL-5 and IL-13 responses following nasal allergen challenge (101). Inhalation of IL-4 increases levels of airways eosinophils (102). Intranasal administration of IL-4 to nonallergic individuals increased levels of IL-6, eosinophils, and neutrophils in nasal lavage fluid 24 hours following challenge (103). This is an important study as a sign of allergic inflammation, increased eosinophils, was seen in nonallergic individuals. Unfortunately this study did not collect any samples at earlier time points, and it would have been very interesting to see if there was any induction of cytokines and chemokines during the early phase.

3.2.3 Aims

The aim of this study is to determine if nasal allergen challenge is a repeatable technique for the technique of cytokine and chemokine detection in nasal lavage fluid and nasal filter paper (FILT P) samples. The dose response characteristic of nasal allergen challenge is also determined.

3.3 Methods

3.3.1 Subjects

The study was approved by the Ethics Committee of The Royal Brompton and Harefield NHS Trust, and all subjects gave written informed consent. 2 cohorts of 8 subjects were recruited for the repeatability and dose response arms of the
trial. The study cohort consisted of male and female, non-smoking subjects with a history of seasonal (intermittent) atopic rhinitis, aged between 18 and 40 years. All subjects had a positive skin prick test to Timothy grass pollen (wheal difference Timothy grass pollen – negative control ≥ 2mm). Subjects were studied outside of the UK hay fever season, i.e. not between May and July. The subjects were all otherwise healthy with no medical problems that might have risked their participation in the study. All subjects had no history of other significant allergies or any respiratory disease other than a history of mild stable asthma not requiring treatment and associated with normal lung function. All subjects had a physical examination with particular attention to the nose to exclude any subjects with structural nasal abnormalities or nasal polyps. Subjects were excluded if they had had an upper respiratory tract infection in the week prior to screening. All subjects were non-smokers and not taking any regular medication. Subjects were not allowed to take any medication for the duration of the trial apart from paracetamol. Table 3.1 below shows the demographic details of the subjects in each group.
Table 3-1 Subject demographics

<table>
<thead>
<tr>
<th></th>
<th>Repeatability</th>
<th>Dose response</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Age (median and range)</td>
<td>24 (20-28)</td>
<td>23 (20-39)</td>
</tr>
<tr>
<td>Gender M:F</td>
<td>5:3</td>
<td>3:5</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caucasians 6</td>
<td>Caucasians 6</td>
</tr>
<tr>
<td>Black</td>
<td>0</td>
<td>Black 1</td>
</tr>
<tr>
<td>Asian</td>
<td>1</td>
<td>Asian 0</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1</td>
<td>Hispanic 0</td>
</tr>
<tr>
<td>Oriental</td>
<td>0</td>
<td>Oriental 1</td>
</tr>
</tbody>
</table>

3.3.2 Nasal challenge

Subjects received their nasal challenge using a Bidose delivery device (Valois, UK) which delivers 100μl of allergen solution to each nostril. The nasal allergen challenge was performed with freeze-dried extract of Timothy grass pollen (Aquagen, *phleum pratense*, ALK freeze dried extract). For the repeatability part of the study total dose of 1000 biological units was given as 500BU to each nostril (0.33µg *Phleum P5* antigen). For the dose response part of the study doses of 50BU (0.033µg *Phleum P5* antigen), 250BU (0.165µg *Phleum P5* antigen) and 500BU (0.33µg *Phleum P5* antigen) were given. The Timothy grass pollen extract was initially dissolved in a diluent solution containing phenol before being dissolved in physiological saline to achieve the final concentration.

3.3.3 Nasal procedures

Nasal symptom scores were carried out as detailed in section 2.1.5 using the nasal symptom score card shown in the appendix, the subjects gave a score of 0-3 for each of the 4 symptom questions giving a maximum score of 12. Subjects were excluded if they had a nasal symptom score of > 2 at baseline. Nasal lavage was carried out using the modified pool technique as detailed in section 2.1.6. The nasal lavage was processed to obtain a cytospin preparation for cell analysis and a supernatant sample was reserved for future cytokine
analysis. Filter paper samples were collected using Whatman 42 filter paper as described in section 2.1.7 and the eluates reserved for future cytokine analysis.

### 3.3.4 Mediator detection

Cytokines in nasal filter paper eluates and nasal lavage were detected using the highly sensitive multiplex Luminex® bead assay as described in section 2.2.3. A standard curve was run in duplicate. The detection limits are shown in the table below.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Nasal filter paper eluates</th>
<th>Nasal lavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>2.14</td>
<td>5.31</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.13</td>
<td>4.66</td>
</tr>
<tr>
<td>IL-13</td>
<td>3.52</td>
<td>4.58</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>10.14</td>
<td>23.3</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.47</td>
<td>3.29</td>
</tr>
</tbody>
</table>

The measurement of PGD₂ and complement factor C3a on in filter paper eluates was by commercially available assay kits. Samples for PGD₂ required treatment with methyl oximating agent to stabilise PGD₂ which was added in accordance with the assay kit manufacturers' recommendations immediately after thawing.

### 3.3.5 Study schedule

All subjects were studied outside of the pollen season. 8 subjects received nasal challenge with 500BU of Timothy grass pollen on 2 consecutive visits with a 2 week washout period. A separate cohort of 8 subjects with seasonal allergic rhinitis were challenged with 50BU, 250BU, and 500BU Timothy grass pollen in a randomly assigned order with a 2 week washout period between each.
3.3.6 Statistical analysis

The data was assessed for normality by assessing data distributions and also comparing the mean and median of the data sets. Formal statistical testing of normality was not carried out as small sample sizes almost always pass a normality test. Since there were frequent non-detectable levels of mediators, the data was not normally distributed; hence box-whisker plots, which show the median, interquartile ranges and the range of the data, represent the data. Plots are also shown of individual subject data, where there is consistency of a colour for a particular subject throughout the graphs. This enables the signal for each cytokine to be compared for each individual subject, and allows response rates to challenge to be assessed

Initially the results from Visit 1 were analysed to determine if there had been a response following NAC by comparing the response at individual time point compared to baseline using the Wilcoxon matched pairs test. If a significant increase was found following challenge then repeatability was assessed. Intra-subject repeatability between 2 visits following NAC with FILT P and nasal lavage sampling was assessed by the calculation of the median % change in Area Under the Curve (AUC) between visits. This is the percentage change from the minimum to the maximum (maximum-minimum + maximum, as a percentage). This was calculated for the early phase and the late phase at TNSS, eosinophils and early phase mediators and cytokines and chemokines for each of the 8 subjects and a median percentage change (with range) was expressed as the summary statistic. The direction (+/-) of this change was not
considered in the analysis of repeatability, since this will distort the median value. When the AUC was zero at both visits, these results were excluded from the analysis. The early phase was considered to be from time 0 to 30 min (inclusive) post NAC, while the late phase was taken from 120 to 480 minutes (inclusive) post NAC. The Bland-Altman method of repeatability measures the coefficient of variability between measures by comparing the average of the difference between the two measures. Initially the bias is calculated which is the difference between the two measures, if the average of this bias is not close to zero it indicates that the results are different on the two occasions. The standard deviation of the differences is used to calculate the limits of agreement which are $1.96 \times SD$ on either side of the mean (339;340). Bland-Altman characteristics are shown for the symptom response and PGD2 in filter paper samples obtained at visit 1.

In the dose response arm differences between doses were tested using the Kruskal-Wallis non-parametric ANOVA test comparing all 3 doses. A p-value of <0.05 was considered significant.

3.4 Results

The following graphs show the results obtained following nasal allergen challenge

Figures 3-1 and 3-2 show the results for nasal symptoms and eosinophils in nasal lavage.

Figures 3-3 and 3-4 show the results for PGD2 and C3a.

Figures 3-5 – 3-7 show the results for IL-4.

Figures 3-8 – 3-10 show the results for IL-5.
Figures 3-11 – 3-13 shows the results for IL-13.

Figures 3-14 and 3-15 show the results for eotaxin IFN-γ.
Figure 3-1 Repeat challenge with 500BU Timothy grass pollen. Effects on nasal symptom scores and eosinophils in nasal lavage. Data shown as median, interquartile range and range. There was an increase in symptoms that was maximal at 15 minutes and had returned to baseline by 480 minutes. Only one subject had an eosinophil response in nasal lavage.
Figure 3-2 Dose response effects following challenge with 50BU, 250BU and 500BU Timothy grass pollen on nasal symptoms and eosinophils in nasal lavage. Data shown as median and interquartile range. Dose response effects for nasal symptoms were seen in the late phase but this did not achieve statistical significance. 500BU Timothy grass pollen was required to produce an increase in eosinophils in nasal lavage.
Figure 3-3 Repeat challenge with 500BU Timothy grass pollen. Effect on PGD₂ and C3a in nasal filter paper eluate. Data shown as median, interquartile range and range. Levels of PGD₂ peak at 5 and 10 minutes. Levels of C3a peak between 5 and 30 minutes. There is also an increase in C3a at 480 minutes.
Figure 3-4 Dose response effects following challenge with 50BU, 250BU and 500BU Timothy grass pollen on PGD2 and C3a. Data shown as median and interquartile range. Early and late phase dose effects were seen with PGD2 and C3a but these did not achieve statistical significance.
Figure 3-5 Repeat challenge with 500BU Timothy grass. Effects on IL-4 in nasal filter paper eluate. Data shown as median, interquartile range and range. There was a significant increase at Visit 1 at 45 minutes.
Figure 3-6 Repeat challenge with 500BU Timothy grass. Effects on IL-4 in nasal lavage. Data shown as median, interquartile range and range. There were significant increases at Visit 1 at 5 – 45 minutes.
Figure 3-7 Dose response effects following challenge with 50BU, 250BU and 500BU Timothy grass pollen on IL-4. Date shown as median and interquartile range.
Figure 3-8 Repeat challenge with 500BU Timothy grass pollen. Effect on IL-5 in filter paper eluate. Data shown as median, interquartile range and range. There were non-significant increases following challenge at Visit 1.
Figure 3-9: Repeat challenge with 500BU Timothy grass pollen. Effect on IL-5 in nasal lavage. Data shown as median, interquartile range and range. There were significant increases at 30 and 45 minutes at Visit 1.
Figure 3-10 Dose response effects following challenge with 50BU, 250BU and 500BU Timothy grass pollen on IL-5. Data shown as median and interquartile range.
Figure 3-11 Repeat challenge with 500BU Timothy grass pollen. Effect on IL-13 in nasal filter paper eluate. Data shown as median, interquartile range and range. There were no increases in IL-13 following nasal allergen challenge.
Figure 3-12 Repeat challenge with 500BU Timothy grass pollen. Effect on IL-13 in nasal lavage. Data shown as median, interquartile range and range. There were increased levels following challenge that were significant at 60 minutes post challenge.
Figure 3-13 Dose response effects following challenge with 50BU, 250BU and 500BU Timothy grass pollen on IL-13. Date shown as median and interquartile range.
Figure 3-14 Repeat challenge with 500BU Timothy grass pollen. Effects on eotaxin and IFN-γ in nasal filter paper eluates. Data shown as median, interquartile range and range. There were no increases in eotaxin and IFN-γ following challenge.
Figure 3-15 Repeat challenge with 500BU Timothy grass pollen. Effects on eotaxin and IFN-γ in nasal lavage. Data shown as median, interquartile range and range. There were significant increases in eotaxin and IFN-γ at 45 and 60 minutes following challenge.
Table 3-3 Results of Wilcoxon matched pairs test (p values) at time points following allergen challenge at visit 1 compared to baseline showing statistically significant results

<table>
<thead>
<tr>
<th>Time point</th>
<th>Symptoms</th>
<th>Eosinophils</th>
<th>PGD₂</th>
<th>C3a</th>
<th>IL-4 FILT P</th>
<th>IL-4 lavage</th>
<th>IL-5 FILT P</th>
<th>IL-5 lavage</th>
<th>IL-13 FILT P</th>
<th>IL-13 lavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>45</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>480</td>
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<td>0.01</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Symptoms</td>
<td>PGD_2</td>
<td>C3a</td>
<td>IL-4 FILT P</td>
<td>IL-4 lavage</td>
<td>IL-5 lavage</td>
<td>IL-13 lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>-------------</td>
<td>-------------</td>
<td>--------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early phase</td>
<td>23 (8-32)</td>
<td>57 (28-96)</td>
<td>39 (3-76)</td>
<td>46 (0-86)</td>
<td>44 (16-74)</td>
<td>32 (5-100)</td>
<td>16 (2-61)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Late phase</td>
<td>69 (9-100)</td>
<td>60 (1-85)</td>
<td>44 (2-75)</td>
<td>37 (10-56)</td>
<td>48 (27-100)</td>
<td>100 (34-100)</td>
<td>50 (25-85)</td>
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</tr>
</tbody>
</table>
### Table 3-5 Bland-Altman data on symptom scores

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Bias</th>
<th>Limits of agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>5</td>
<td>-0.62</td>
<td>-5.30</td>
</tr>
<tr>
<td>10</td>
<td>0.25</td>
<td>-5.17</td>
</tr>
<tr>
<td>15</td>
<td>-0.12</td>
<td>-4.50</td>
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<tr>
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<td>-2.70</td>
</tr>
<tr>
<td>480</td>
<td>-0.25</td>
<td>-1.56</td>
</tr>
</tbody>
</table>

### Table 3-6 Bland Altman data on PGD2 in filterpaper eluate

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Bias</th>
<th>Limits of agreement</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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<td>-20.9</td>
</tr>
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<td>5</td>
<td>22.19</td>
<td>-239.08</td>
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<tr>
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<td>-10.06</td>
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<td>-33.77</td>
</tr>
<tr>
<td>480</td>
<td>9.10</td>
<td>-22.08</td>
</tr>
</tbody>
</table>
3.4.1 Results summary

3.4.1.1 Nasal symptom scores and eosinophils in nasal lavage

Following nasal allergen challenge there was an immediate increase in symptoms that was maximal up to 15 minutes and had returned to baseline by 8 hours following challenge. Bland-Altman analysis showed that symptom scores were repeatable. Dose response effects were seen in the symptom scores and this achieved statistical significance (p=0.042). There was no significant increase in eosinophils in nasal lavage following challenge with 500 BU of Timothy grass. Repeat challenge with 500 BU of Timothy grass resulted in a median absolute change (range) in AUC from visit 1 to visit 2 of 23% (8-32) for total nasal symptom scores during early response (0-30 min). For late phase (120-480 minutes) responses, the median absolute change (range) in AUC from visit 1 to visit 2 was 69% (9-100). Dose response effects were not seen for levels of eosinophils in nasal lavage following NAC.

3.4.1.2 Early phase mediators

Levels of PGD2 detected in filter paper (FILT P) eluates peaked at 5 and 10 minutes post NAC, and for C3a the peak is seen between 5 and 30 minutes, and again at 480 minutes. Repeat challenge with 500 BU of Timothy grass resulted in a median absolute change (range) in AUC from visit 1 to visit 2 of 57 (28-96) for PGD2 and 39 (3-76) for C3a as sampled by FILT P during early response. Bland-Altman analysis showed that measured of PGD2 was poorly repeatable. Dose response effects for PGD2 and C3a were seen during both the early and the late phase responses and this achieved statistical significance for C3a in the early phase (0-30 minutes), (p=0.01).
Levels of cytokines and chemokines detected in filter paper (FILT P) eluates were generally higher than those in nasal lavage.

There was a significant increase in IL-4 in lavage during the early phase and for IL-5 in nasal lavage at the end of the early phase. There was a significant increase in IL-13 in nasal lavage at 60 minutes post challenge.

The filter paper eluates did not yield increases in cytokine levels, with no clear early or late responses and dose response calculations were not made. Levels of IL-4 were generally <50pg/ml (Fig 3-5) levels of IL-5 were generally <20pg/ml (Fig 3-8), and IL-13 levels <150pg/ml (Fig 3-8). The same individual had high levels of IL-4, IL-5 and IL-13 in nasal FILT P eluate at 45 min. Repeat challenge with 500 BU of Timothy grass resulted in a median absolute change (range) in AUC from visit 1 to visit 2 of 44 (16-74) for IL-4, 32 (5-100) for IL-5 and 16 (2-61) for IL-13 as sampled by nasal lavage during the early phase. Repeat challenge with 500 BU of Timothy grass resulted in a median absolute change (range) in AUC from visit 1 to visit 2 of 48 (27-100) for IL-4, 100 (34-100) for IL-5 and 50 (25-85) for IL-13 as sampled by nasal lavage during the late phase.

### 3.5 Discussion

A single nasal allergen challenge (NAC) model is potentially a very efficient study design for drug development as it is possible to obtain serial measurements of biomarkers that relate to inflammation in the eight hours following challenge (68), which is not a feature of inhaled or skin allergen challenge. In this NAC model we measure symptoms, levels of lavage eosinophils, and our primary end-point was to focus especially on late phase levels of IL-4, IL-5 and IL-13 measured in FILT P eluates.
However, in the current repeat part of the nasal allergen challenge (NAC), that employed the higher allergen dose of 500BU, in the 8 individuals there were no significant changes in levels of IL-4, IL-5 and IL-13 at 2h, 4h, 6h and 8h when comparing with baseline for nasal lavages and for filter paper eluates. This was despite subjects having symptomatic responses to grass pollen challenge.

This failure to detect significance in the current study may partly have been due to only 8 subjects being studied, and these being low producers of IL-4, IL-5 and IL-13 in the late phase. However, it is important to stress that in our study levels of these cytokines in FILT P eluate were very variable and were generally higher in terms of medians compared to a previous study that measured FILT P eluates (68). The failure to elicit a significant response in terms of levels of IL-4, IL-5 and IL-13 with the high 500BU dose renders assessment of a dose response invalid.

It is important to compare these results with those previously found using this NAC model from our group. In 3 published studies from the NHLI Clinical Studies Unit this NAC model has been found to cause significant changes in levels of IL-5 in nasal lavage in the late phase (4 to 8h post NAC) when comparing with baseline. The first of these studies employed a single dose of topical nasal corticosteroid (101), and measured nasal lavage IL-5 and IL-13 in 32 subjects. The second study employed 7 days of dosing with a topical corticosteroid (100); and measured IL-4, IL-5 and IL-13 in both nasal lavages and filter paper eluates in 13 subjects. Finally, a single dose of a topical combined beta-tryptase/trypsin inhibitor (50) was used in 16 subjects to assess levels of IL-5 in nasal lavage fluid. In all 3 of these studies significant changes in levels of cytokines (IL-5 with sometimes IL-4 and IL-13) were detected in the
late phase when comparing with baseline. In addition, in the 3 studies significant effects of therapy on these mediators were noted compared to placebo.

Of the 3 studies, the study with 7 days of nasal corticosteroid was the only one to assess levels of IL-4, IL-5 and IL-13 in both nasal lavages and eluates from Whatman’s filter paper number 42 (100). This study established that higher levels of all 3 of these mediators were detectable in filter paper eluates compared to nasal lavages (Figure 2 of (100). However, when scrutinising data for individual subjects from this study (poster at the American Thoracic Society) it was noted that high levels of IL-5 and IL-13 were detected in filter paper eluates in just 2 of 13 subjects. In this situation, there is the need to use non-parametric methodology to analyse and present the data. In graphical presentation this should have involved whisker plots to show median/interquartile range/range (non-parametric data summary statistics), rather than mean and SEM (parametric data summary statistics). Hence it could be argued that the results achieved in this study depended on those 2 individuals that were responders in terms of IL-4, IL-5 and IL-13 in nasal filter paper eluates. Therefore, another reason why we failed to detect significant changes in levels of IL-4, IL-5 and IL-13 in the late phase may have been because we did not recruit “responders” in terms of IL-4, IL-5 and IL-13.

Despite not seeing changes in levels of IL-4, IL-5 and IL-13 after NAC; it was interesting to note that a single subject with a very high eosinophil response also had high IL-5 levels in the late phase suggesting a possible mechanism for the appearance of eosinophils in nasal lavage. In addition, 2 subjects (numbers
had higher levels of IL-5 in the late phase measured in nasal filter paper and nasal lavage on both visits.

In the current study, complement factor 3a (C3a) and prostaglandin D2 (PGD2) were significantly increased from baseline in the acute phase (up to 60 min) for both nasal lavage and nasal filter paper levels. PGD2 levels peaked at 5-10 mins, and C3a peaked at between 5 and 30 min. The increase of mediators in the early phase may be due to the release of pre-formed mediators from cells such as degranulating mast cells, and PGD2 can be rapidly synthesised through the arachidonic acid pathway. In contrast, increase in interleukin levels during the late phase could represent new synthesis of protein. The early phase mediators PGD2 and C3a are poorly repeatable and large sample sizes would be needed to detect changes in response to treatment. The variability in these mediators could be due to the presence of proteases in nasal secretions, and this in an area that could be investigated in future studies. The early phase C3a levels varied with the allergen dose, but the upper dose level for allergen was not defined in this study. Further studies should be carried out with higher doses of allergen, as this may lead to increased numbers of responders to challenge. There was considerable inter-subject variability that was consistent over the 2 visits.

The choice of dose for allergen challenge studies is clearly of crucial importance. With regard to inhaled allergen challenge there is a careful balance between a dose high enough to produce a response but no so high as to produce a dangerous reduction in FEV1. Using skin prick titration in combination with PC20 for histamine has been shown to be useful for guiding the dose of inhaled allergen challenge (341;342). However it has been shown
that whilst there is a correlation between skin test threshold and inhaled response in some subjects this relationship is inconsistent (343). In a study comparing responses in the nose following house dust mite challenge, the wheal diameter following skin prick challenge correlated with the nasal allergen challenge threshold (344). However by the using the nasal allergen challenge method the concern about maximal dose is not as great as with inhaled challenge with regards to fall in lung function. However we would not wish to use a supramaximal dose as this may not be able to be overcome by any of the drugs under study. The dose used in the studies presented in this Thesis is based on the work of Professor Durham, although his group apply the allergen to the nose using paper disks rather than the nasal spray used in these studies. A dose of 500BU given by nasal spray has also been shown to cause consistent symptom responses in three published studies (50;100;101).

In addition to recording symptoms another physiological measure of nasal response following allergen challenge is rhinomanometry which is a measure of the physical changes that occur post allergen challenge. Nasal obstruction can be measured by anterior and posterior rhinomanometry and is generally accepted as the standard measure of nasal airways resistance (345). Other methods used to measure nasal patency include acoustic rhinometry (346), and CT scanning. (347). Nasal peak flow is a very simple method for assessing nasal patency but does have the disadvantage of also being affected by changes in pulmonary function(348). These methods could be utilised in future studies to guide the allergen dose, by selecting a dose for an individual patient that caused a 50% reduction in the airway on objective monitoring and then using this dose when measuring cytokine levels.
In this study the levels of mediators in filter paper eluates were not corrected for the weight of the sample obtained as the original methodology paper does not suggest that this is necessary (329). Similarly the levels of mediators in nasal lavage were not corrected for the volume of nasal lavage recovered as nasal lavage recovery volumes were generally consistent (98).

The levels of mediators were higher in nasal filter paper eluates than in nasal lavage and it likely that this is due to the dilutional effect of the 5.0mls of saline employed in nasal lavage. However it is important to recognise that the filter paper eluates may represent different signals. The presence of filter paper may lead to cells becoming adherent and undergoing lysis and hence giving a signal of intracellular and extracellular mediators. However during collection of lavage there should not be any cell lysis and hence the detectable levels should represent only the extracellular component of the signal. Both nasal filter paper eluate and lavage are dependent on the mediators being soluble and released into nasal epithelial lining fluid. Where tissue analysis is required samples can be obtained by biopsy, curettage or brushing.

A comparative analysis technique was employed to consider the factors that may have caused the failure to see significant increases in levels of IL-4, IL-5 and IL-13 in the late phase filter paper eluates when comparing with the published studies (100) (101) (50). In this analysis all the potential causes are first listed, and then the most likely factors considered, so that those causative factors can then potentially be remedied.
Table 3-7. Potential Factors to Account for the Failure to Detect Significant Increases in Late Phase Nasal Filter Paper levels of IL-4, IL-5, and IL-13 after Nasal Allergen Challenge (NAC). Single dose NAC and repeat dose NAC studies were compared to a published study (349).

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<thead>
<tr>
<th>Study Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>Different hay fever sufferers, out of season, with Timothy grass pollen sensitivity (confirmed by skin prick test) were used in the 3 studies. In the published study only 2 of 8 subjects had prominent IL-4 and IL-13 responses, and it is possible that in the more recent studies there were less responders.</td>
</tr>
<tr>
<td>Nasal delivery device</td>
<td>There was a change from the Bidose (Valois) to the Dolphin (Valois) device from the single dose to the 4 day repeat NAC model. However, this is unlikely to be a factor: since both devices actuate reliably when checking by weight, and the vials and stoppers that contain the actual allergen within the device are identical.</td>
</tr>
<tr>
<td>Timothy Grass Pollen allergen</td>
<td>Aquagen SQ Timothy Grass Pollen (ALK, Denmark) (500 BU/nostril) was used in both studies, always within the manufacturer's expiry date. However, different allergen batches were used, and the allergen dose may be too low. The potency of the allergen is established by the manufacturer (ALK) on in vitro assays, and it would be ideal to have a full dose response for NAC with each batch in patients. In a given study a single batch of allergen should be employed.</td>
</tr>
<tr>
<td>Diluents used for Timothy Grass allergen for NAC</td>
<td>Manufacturer's (ALK) diluent (with 1% phenol) and saline (0.9%) was used in identical reconstitution protocols in both studies</td>
</tr>
<tr>
<td>Storage of dilute allergen in nasal delivery device</td>
<td>The diluent was stored at 4°degrees C for up to 4 weeks in stoppered vials in all studies.</td>
</tr>
<tr>
<td>Clinicians performing procedure</td>
<td>Ed Erin took part in the published study, while I (Helen Neighbour) carried out the single dose and repeat dose NACs.</td>
</tr>
<tr>
<td>Application of nasal paper, and paper size</td>
<td>The same filter paper size (30mm by 7mm) and same application time (2 min) were employed.</td>
</tr>
<tr>
<td>Clinical response assessed by total nasal symptom scores (TNSS)</td>
<td>TNSS levels were as expected across the studies, with early and late phase response observed in subjects on no drug (study 1) or placebo (study 2), and reduced response in the active treatment group (study 2)</td>
</tr>
</tbody>
</table>

LABORATORY PHASE

Page 116 of 293
Ed Erin performed the processing of samples in the published study, while I and Grant Nicholson processed them in the single and repeat dose NAC studies.

Whatman's No 42 filter paper was used in both studies, as specified in the study protocol, but different batches were employed.

The same elution buffer was used in both studies, namely Beadlyte Assay Buffer, containing BSA 1%, azide, tween 20 Supplied by Upstate, 20ml (CAT 43-002)

Bovine serum albumin (BSA) Unchanged between the studies: thus not a factor

Elution methodology Not a factor

Storage of samples Samples were stored at -80°C in both studies, with a single cycle of freeze-thawing.

This was not found to be a factor, on checking filters with control samples. Centrifugal filtration was performed using Corning Filter Membranes in both studies. Ed Erin employed Corning cellulose acetate filters (defined by the manufacturer as very low protein binding) I used Corning nylon filters (defined by the manufacturer as low to moderate protein binding)

Sample tubes and containers Eppendorfs and cryo tubes were used in both studies

Luminex procedure; Including incubation times Identical procedure in both studies

Software to download Luminex for published studies and single dose

Manual checks on downloads Performed

Sample application to microtitre plate Random in both studies

Control curves on Luminex Equivalent

Paired samples Good fit

In summary, a number of elements need to be considered, and some protocol and methodological alterations made in order to be able to detect higher levels of IL-4, IL-5 and IL-13 in nasal lining fluid.
It is important to have sufficient responder subjects, and it might be possible to perform a screening NAC in order to be able to select IL-4/5/13 responders.

A particular concern is that there may be batch variability for Whatman's No. 42 filter paper, especially since this material is not designed to absorb biological fluids and efficiently release the contained proteins. One could either perform spiking experiments to select a batch of Whatman's FILT P that is efficient at releasing proteins, or one could consider alternative synthetic absorptive matrices (SAM). On contacting Pall Gellman, the international manufacturers of electrophoretic media and support material for laboratory protein assays, we found that they had a range of SAMs with specifications for extremely low protein binding. They recommended Accuwik Ultra because this is extensively used in urine pregnancy kits and in automated immunoassay machines to absorb and release reagents containing proteins.

Results are shown in Appendix 2 for further studies performed to assess a SAM in comparison to Whatman's filter paper No. 42, in relation to detectable levels of IL-4, IL-5 and IL-13. I was involved in the design of these experiments, but did not carry out the clinical and laboratory procedures (Ed Erin with Grant Nicholson). The conclusion from this series of experiments was that Whatman's filter paper is inferior to the synthetic absorptive matrix (SAM) (Accuwik Ultra) in terms of absorption and release of nasal lining fluid cytokines. In addition the dose of allergen used for NAC should be increased to increase the likelihood of achieving higher detectable levels of IL-4 IL-5 and IL-13 in the late phase.

In a NAC study carried out on the CSU in the first quarter of 2007 (Linda Brinkman on clinical procedure, Grant Nicholson on laboratory aspects), the dose of allergen was increased from 500BU (0.3μg of *Phleum pratense*...
allergen) to 1.0µg of *Phleum pratense* and the Accuwik Ultra SAM was employed. These modifications enabled high levels of IL-4, IL-5 and IL-13 to be detected in SAM eluates in the late phase: levels were higher than any previously found in studies performed on the CSU. In 8 of 12 individuals, levels of IL-5 and IL-13 were consistently >100pg/ml in the late phase. The raw data is shown in Appendix 2 (Figure 11.4).

### 3.6 Conclusions

1. Single dose NAC causes an increase in nasal symptoms and is safe and well tolerated.

2. There was no late phase response for IL-4, IL-5 and IL-13 in nasal filter paper eluates or nasal lavage samples.

3. The early phase mediators C3a and PGD₂ are poorly repeatable.

4. Whatman’s 42 is not the optimum filter paper for measuring cytokines in nasal secretions.

### 3.7 Future study

1. One should only employ Whatman’s filter paper if it has appropriate elution characteristics for a particular batch demonstrated by spiking with commercial standards and nasal samples. Given the complexities of this approach there is a need to identify and validate novel preferably synthetic absorptive matrices (SAM) with reproducible and efficient elution characteristics.

2. There is still a need to determine the optimum dose of NAC and also in the context of repeat daily NAC.
4 Repeat dose nasal allergen challenge (NAC): development of a model that mimics natural seasonal exposure and exhibits priming

4.1 Abstract

Introduction: Single dose NAC does not reproduce the allergen exposure during the pollen season. Topical nasal steroids have been shown to inhibit the early and late phase reactions, with single doses inhibiting the late nasal reaction, and 6 weeks of therapy inhibiting the early and late nasal reactions.

Aims: To determine if repeated daily nasal allergen challenge will cause sensitisation in terms of increased levels of symptoms, eosinophils and inflammatory mediators.

Methods: A total of 14 patients with Timothy grass pollen seasonal allergic rhinitis were recruited out of season; receiving a total of 6 nasal challenges over a 2 week period, with 4 of these challenges on consecutive days. 8 subjects were challenged with 500BU Timothy grass pollen to each nostril without prior fluticasone, 4 received 100μg fluticasone to each nostril prior to repeat nasal challenges, and 2 subjects received placebo challenge and no therapy. Symptom scores were recorded following nasal challenge, and nasal lavage and nasal filter paper samples were taken for cell and cytokine/chemokine analysis respectively.

Results: Repeated NAC caused a trend for priming in terms of increased symptoms that did not reach significance. With repeated NAC there was an increase in levels of eosinophils detectable in nasal lavage at 1h at visits 5, 6 and 7 (p<0.05). There was evidence of priming for IL-5 levels in nasal lavage at
1h at visit 5 and 6h at visit 9 ($p<0.01$). In addition, levels of IL-13 in nasal lavage showed evidence of priming at 6h post NAC at visit 7 and 9 ($p<0.01$). Of the 4 individuals in the fluticasone arm, there were very low responses to NAC at baseline, and priming and effects of fluticasone could not be commented on. For the 2 individuals receiving placebo challenge there was no symptomatic or eosinophil response, and levels of lavage chemokines and cytokines were generally very low.

**Conclusions:** This study of repeat NAC on 4 consecutive days demonstrated a trend for increased symptoms, with evidence of priming in terms of increased levels of eosinophils, and nasal lavage IL-5 and IL-13. Levels of chemokines and cytokines in nasal FILT P eluate were low and this may be due to batch variability in Whatman's filter paper, with potential for cytokines and chemokines binding to the paper and failing to elute. Effects of topical nasal fluticasone could not be seen, while placebo challenge did not cause symptoms nor appreciable eosinophil or chemokine/cytokine responses.

### 4.2 Introduction

#### 4.2.1 Use of nasal allergen challenge

The use of a nasal challenge model has several advantages over inhaled allergen challenge including ease of recruitment, subject safety and ability to obtain samples at multiple time points. However the single dose challenge model has limitations; since it does not accurately model the exposure that occurs during the hay fever season. The aim of the model developed in this Chapter aims to replicate seasonal exposure to allergen by giving NAC on 4 consecutive days and hence more accurately model the changes that occur in seasonal allergic rhinitis. It is also hoped that there will be priming and hence
increased levels of cytokine will be detected. It would be possible to challenge subjects with seasonal allergic rhinitis during the allergen season during which the nasal mucosa will be primed however it is not possible to quantify the exact level of allergen exposure and hence is difficult to make valid comparisons. The higher the level of cytokines following allergen challenge, the more likely an effect of a novel therapeutic will be statistically significant.

The Vienna chamber is the challenge model that most accurately mimics the allergen exposure in seasonal allergic rhinitis generally for a period of up to 8 hours. 6 to 8 subjects with hay fever are placed in a sealed room outside the grass pollen season, and are challenged with an accurately quantifiable amount of allergen which is added via the air-conditioning system with a proven homogenous distribution of pollen grains, in conditions of allergen exposure that mimic those of a hot summer day. (350). This NAC can then be repeated over several days. Generally, subjects remain within the Vienna chamber, but samples can be obtained from the subjects at set time points following the challenge.

In a variation of this form of challenge, Environmental Challenge Rooms can seat up to 100 subjects, and allergens are delivered via nebulisers within the room (Applied Clinical Research, Toronto). One of the advantages is that it allows a large number of subjects to be challenged with the same dose of allergen at the same time. However, repeated sampling with rapid processing of specimens is not practical in this context. The nasal spray challenge model shown here can be carried in smaller research units as subjects can be challenged individually and a series of samples processed immediately.
4.2.2 Priming

Priming is the increased levels of inflammation seen following repeated allergen exposure compared with a single large dose of allergen (351);(352). There is a cumulative effect of serial challenges. Repetitive allergen challenge might cause an accumulation of inflammatory cells and their mediators in the nasal mucosa and hence increase the response to subsequent challenges. It has been shown that local accumulation of eosinophils, neutrophils and mononuclear cells persists for 48 hours and is accompanied by release of ECP, MPO, LTB₄ and histamine. (353). Additionally there might be effects on circulating cells or the airway epithelial cells themselves that might modify subsequent responses. In contrast to effect of repeat challenge on airways mucosa there is no increase in response to repeated allergen challenge using skin prick testing. However if the allergen in injected into the skin then priming is seen (324). These findings may indicate that the priming seen in repeat airways challenge may be due to changes in the epithelial barrier function (354). It has been shown that unilateral repeated nasal allergen challenge causes priming, that is not seen in the contralateral nostril suggesting that priming is due to changes in the local mucosa rather than a systemic effect (351).

Priming also occurs during seasonal exposure to allergen, and this effect persists for several weeks following the end of the allergen season (352). Priming of the symptom response has been demonstrated in the nasal mucosa using a ragweed model of seasonal allergic rhinitis. There is an influx of inflammatory cells prior to each of the challenges (355). The use of the Vienna chamber has shown that a specific nasal response in increased by environmental priming, as demonstrated by exposing grass pollen sensitive
individuals with other sensitivities to the allergen other than grass pollen to which they are sensitive and then challenging with grass pollen. There was augmentation of the response to grass pollen challenge following prior challenge with other allergens (356). Eosinophils release major basic protein (MBP) and other granule proteins, which can damage the mucosal barrier which may allow the allergen to reach submucosal cells more readily (357). MBP can also reduce ciliary activity and hence increase the time the nasal mucosa is exposed to pollen allergens (358).

4.2.3 Repeated allergen challenge

Studies of the consequence of repeated allergen challenge and the effect of priming have been carried out previously. A study comparing the effect of a large single allergen challenge compared with the same dose spread over 4 days on the inflammation seen in asthmatic airways showed that 2 consecutive days of lower dose allergen produced the same degree of sputum eosinophilia as a single large dose challenge (359). Studies have shown that the priming response in the nose to allergen is specific to the side of the nose challenged, disappears when the exposure is stopped, occurs during seasonal exposure and is not specific for the antigen used (351). Nasal responses to repeated doses of ragweed have been determined by measuring levels of histamine, TAME-esterase activity and kinins in nasal lavage fluid and by symptom scores. 92.7% of subjects had an early phase reaction in both mediators and symptoms, 47% of subjects developed a late increase in symptoms. The levels of late phase mediators could not be predicted by skin test response or serum IgE measurement. On re-challenging subjects 21% had an increase in mediator levels during the early phase reaction (360). In birch pollen allergic subjects it
has been shown that repeated nasal challenge over 7 days resulted in a gradual increase in nasal symptoms. There was also a priming effect on the number of eosinophils in the nasal mucosa, and the levels of eosinophils correlated with the symptom score (361).

Interestingly when non-asthmatic subjects with allergic rhinitis received inhaled allergen challenge there was an increase in eosinophils in induced sputum without any in respiratory symptoms, and with repeated challenge priming was not observed. However the doses used in this study were significantly lower than those used in other studies (362). The time interval required for priming has also been assessed, when subjects received nasal allergen challenge (NAC) 3 days, 1, 2 and 4 weeks following initial nasal challenge. The 3 day interval group showed an increased response, the 1 and 4 week interval group showed a similar response to baseline; while the 2 week group showed a reduced response when compared to baseline (363). The reduced response seen at 2 weeks may involve a mechanism similar to that of immunotherapy.

It has also been shown that placebo nasal challenge does not cause nasal symptoms or decrease in nasal patency in allergic subjects (352). This suggests that the changes seen following allergen challenge are immune mediated rather than due to physical effects of nasal challenge. Repeat allergen challenge has also been studied in asthmatic airways. Repeated inhaled allergen challenge causes airway eosinophilia and increased IL-5, associated with airway hyper-responsiveness as determined by methacholine challenge without the development of marked acute bronchoconstriction or the development of a late response (364).
A cumulative dose trial of inhaled allergen challenge in asthmatics has shown an increase in IL-4 IL-5 and IL-13 mRNA expression in bronchoalveolar cells. (365;366). This increase in cytokines is a possible mechanism for the priming seen in repeat dose allergen challenge models. In addition eosinophils form subjects with allergic asthma exhibit priming, probably as a result of their interaction with cytokines in peripheral blood. The priming enhances migration into the airways due to increased chemotaxis and endothelial adhesion response. (367-370).

The priming effect in skin has be used to test a novel therapeutic agent, the anti-IgE Omalizumab prevents the repeat dose priming effect of increased inflammatory cells in skin with repeated allergen challenge (325). This suggests that a model of allergen priming can be used to test a novel therapeutic agent.

4.2.4 Effects of steroids on nasal response to repeated allergen challenge

This study also investigates the effects of a topical nasal steroid, fluticasone, on repeat dose allergen. When budesonide is given 30 minutes prior to allergen challenge there was no effect on nasal symptoms, but nasal filter paper eluate levels of GM-CSF and IL-5 were inhibited during the late phase (371). In a repeat dose allergen model budesonide has been shown to inhibit the increase in symptoms and also levels of TAME in nasal lavage (372). It has been shown that repeated nasal allergen challenge can increase levels of eotaxin in nasal lavage and this can be reduced by topical budesonide started 2 weeks prior to first challenge (373). Pre-treatment with topical nasal steroids for a week prior to challenge decreased symptom scores and levels of histamine, TAME, and kinins in the early, late and re challenge reactions (374).
Topical nasal budesonide has been shown to reduce early phase symptoms if started for a week prior to a repeat dose allergen challenge study (375). Topical steroids have also been shown to reduce the early reduction in nasal peak inspiratory flow in a repeat dose allergen challenge study (376). However oral glucocorticoids inhibit the late phase of an allergic reaction but not the early phase (377). Inhaled budesonide has been shown to reduce allergen induced sputum eosinophilia and at higher doses reduces airway hyperresponsiveness following repeat low dose allergen challenge in atopic asthmatics (378).

4.2.5 Aims

The aim of this study was to develop a nasal allergen challenge model that more closely resembles the natural pollen exposure that occurs during the hay fever season and leads to increased levels of cytokines in nasal filter paper eluates.

4.3 Methods

4.3.1 Subjects

The study was approved by the Ethics Committee of The Royal Brompton and Harefield NHS Trust, and all subjects gave written informed consent. The study cohort consisted of male and female, non-smoking subjects with a history of seasonal (intermittent) allergic rhinitis, aged between 18 and 40 years. All subjects had a positive skin prick test to Timothy grass pollen (wheal difference Timothy grass pollen – negative control ≥ 3 mm). Subjects were studied outside the UK hay fever season, outside May to July. The subjects were all otherwise healthy with no medical problems that might have risked their participation in the study. All subjects had no history of other significant allergies as environmental priming has been described (356). The subjects did not have
respiratory disease other than a history of mild stable asthma not requiring treatment and associated with normal lung function. All subjects had a physical examination with particular attention to the nose to exclude any subjects with structural nasal abnormalities or nasal polyps. Subjects were excluded if they had had an upper respiratory tract infection in the week prior to screening. All subjects were non-smokers and not taking any regular medication. Subjects were not allowed to take any medication for the duration of the trial apart from paracetamol.

Table 4.1 below shows the demographic and baseline details of the subjects in each group. Data is represented as the median and the range. As the placebo group only contains 2 subjects individual patient data is shown.
Table 4-1 - Subject demographics

<table>
<thead>
<tr>
<th></th>
<th>Allergen</th>
<th>Fluticasone</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Age</td>
<td>26 (20-40)</td>
<td>24 (19-26)</td>
<td>22-28</td>
</tr>
<tr>
<td>Gender M:F</td>
<td>4:4</td>
<td>1:3</td>
<td>2:0</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Cauc 5</td>
<td>Cauc 1</td>
<td>Cauc 1</td>
</tr>
<tr>
<td></td>
<td>Afro-caribbean 3</td>
<td>Afro-caribbean 1</td>
<td>Afro-caribbean 1</td>
</tr>
<tr>
<td></td>
<td>Hispanic 1</td>
<td>Hispanic 1</td>
<td>Hispanic 1</td>
</tr>
<tr>
<td>Total IgE</td>
<td>191 (21-788)</td>
<td>142 (43-539)</td>
<td>56, 130</td>
</tr>
<tr>
<td>Timothy grass IgE</td>
<td>35 (6-860)</td>
<td>20 (8-33)</td>
<td>6, 8</td>
</tr>
</tbody>
</table>

4.3.2 Nasal challenge

Subjects received their nasal challenge using a Dolphin delivery device (Valois, UK) which delivers 100μl of allergen solution to each nostril. The nasal allergen challenge was performed with freeze-dried extract of Timothy grass pollen (Aquagen, *phleum pratense*, ALK freeze dried extract). A total dose of 1000 biological units was given as 500BU to each nostril (0.33μg *Phleum P5* antigen). The Timothy grass pollen extract was initially dissolved in a diluent solution containing phenol before being dissolved in physiological saline to achieve the final concentration. Therefore the placebo nasal challenge was made by diluting the same volume of diluent used to dissolve the allergen in the same volume of saline to ensure that the allergen and placebo challenges contained equal amounts of phenol. The fluticasone was administered using a commercially available nasal spray delivering 100μg to each nostril.

4.3.3 Nasal procedures

Nasal symptom scores were carried out as detailed in section 2.1.5 using the nasal symptom score card shown in the appendix, the subjects gave a score of
0-3 for each of the 4 symptom questions giving a maximum score of 12. Subjects were excluded if they had a nasal symptom score of > 2 at baseline. Nasal lavage was carried out using the modified pool technique as detailed in section 2.1.6. The nasal lavage was processed to obtain a cytopsin preparation for cell analysis and a supernatant sample was reserved for future cytokine analysis. Filter paper samples were collected using Whatman 42 filter paper as described in section 2.1.7 and the eluates reserved for future cytokine analysis.

4.3.4 Cytokine detection

Cytokines in nasal filter paper eluates were detected using the highly sensitive multiplex Luminex® bead assay as described in section 2.2.3. A standard curve was run in duplicate and all samples were run in duplicate and the mean taken of the two values. The detection limits for each cytokine are shown in the table below.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Detection limit (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>1.3</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.9</td>
</tr>
<tr>
<td>IL-13</td>
<td>5.4</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>11.7</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>14.6</td>
</tr>
</tbody>
</table>

The cytokine levels in the nasal lavage samples were measured using the Meso Scale Discovery Ultra sensitive cytokine detection assay which has a detection limit of 0.6pg/ml.

4.3.5 Study schedule

Arm 1: Nasal allergen challenge (n= 8)
The schedule, days and visit numbers for the study are presented. The red arrows indicate the timing of an allergen challenge, the letter F indicates when fluticasone was given. The white arrows indicate the timing of placebo challenge. Visits 3, 8 and 10 were performed at 24 hours following the challenge, and symptom score data and nasal filter paper and lavage samples were collected. Nasal filter paper samples and symptom scores were collected prior to challenge and at 15 minutes and 1 and 6 hours post challenge. Nasal lavage samples for eosinophils and cytokine analyses were collected prior to challenge and 1 and 6 hours post challenge. Blood samples for IgE and IL-13 measurement were collected at V2 and V10.
4.3.6 Statistical analysis

The data was assessed for normality by looking at data distributions and also comparing the mean and median of the data sets. Since there were many zero values in the data set, the data was not normally distributed, with the mean and median being disparate. Hence box-whisker plots, which show the median, interquartile ranges and the range of the data, represent the data. Plots are also shown of individual subject data, where there is consistency of a colour for a particular subject throughout the graphs. This enables the signal for each cytokine to be compared for each individual subject, and allows response rates to challenge to be assessed. Priming was determined by comparing responses at the first challenge visit (V2) with the response at the same time point in the subsequent challenge visits (V4, V5, V6, V7, V9) using the Wilcoxon matched pairs test.
4.4 Results

4.4.1 Symptom score responses to nasal challenge with allergen, allergen and fluticasone and placebo challenge

![Graphs showing symptom scores for allergen, Flixotide, and Placebo]

Figure 4-1 Effect of repeated nasal challenge on total nasal symptom score. Data shown as median, interquartile range and range. There was an increase in pre-challenge symptoms between V2 and V7 in the allergen arm, however this did not achieve statistical significance. There was no evidence of priming at 1 or 6 hours post allergen challenge. Administration of fluticasone before allergen challenge did not modify the symptom response to allergen challenge. Placebo challenge did not cause nasal symptoms.
4.4.2 Change in % eosinophils in nasal lavage in response to nasal challenge with allergen, allergen and fluticasone and placebo challenge.

<table>
<thead>
<tr>
<th>Allergen challenge</th>
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</table>

<table>
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<tr>
<th>Flixotide and allergen challenge</th>
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</thead>
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<td></td>
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<table>
<thead>
<tr>
<th>Placebo challenge</th>
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</tr>
</tbody>
</table>

Figure 4-2 Effect of repeated nasal challenge on % eosinophils recovered from nasal lavage. Data shown as median, interquartile range and range.
Repeated nasal allergen challenge caused an increase in the % eosinophils in nasal lavage. There was no evidence of priming at baseline or at 6 hours post challenge with repeated allergen challenge. However at 1 hour post allergen challenge there was a statistically significant increase in the response at V5 (p=0.03), V6 (p=0.03) and V7 (p=0.05), but not visit 9. Fluticasone did not reduce levels of eosinophils in nasal lavage following nasal allergen challenge. Placebo challenge did not increase the % of eosinophils in nasal lavage.
4.4.3 Levels of cytokines in nasal samples following repeated nasal allergen challenge

Figure 4-3 Effect of repeated nasal allergen challenge on IL-4. Data shown as median, interquartile range and range
In general, IL-4 levels are higher in lavage than paper, and it is noted that lavage levels do not seem correlate with paper levels. There was a non-significant increase in IL-4 in nasal lavage at V9 6 hours compared with V2 6 hours in nasal lavage. There was no evidence of priming of the IL-4 response in nasal lavage to repeated allergen challenge.
In general, IL-5 levels are higher in lavage than paper, and it is noted that lavage levels do not seem correlate with paper levels. There was no evidence of priming at baseline. There was evidence of priming in nasal lavage at 1 hour at V5 (p=0.01), however this priming effect was not sustained across the challenge week. There was evidence of priming at 6 hours at V9 (p=0.008).
Figure 4-5 Effect of repeated nasal allergen challenge on IL-13. Data shown as median, interquartile range and range.

In general, IL-13 levels are higher in lavage than paper, and it is noted that lavage levels do not seem correlate with paper levels. There was no evidence of priming at baseline or 1 hour post challenge. There was evidence of priming at 6 hours post challenge at V7 (p=0.01) and V9 (p=0.008).
Figure 4-6 Effect of repeated nasal allergen challenge on eotaxin. Data shown as median, interquartile range and range. There were non-significant increases in eotaxin in nasal lavage following repeated nasal allergen challenge and there was no evidence of priming.
Figure 4-7 Effect of nasal allergen challenge on IFNγ. Data shown as median, interquartile range and range.

There was no increase in nasal lavage levels of IFN-gamma following nasal allergen challenge and there was no evidence of priming.
The following table summarises the effect of repeated nasal allergen challenge on cytokine levels in nasal lavage fluid.

<table>
<thead>
<tr>
<th>Table 4-3 Cytokines in nasal lavage following repeated nasal allergen challenge</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection limit pg/ml</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Magnitude of maximum signal pg/ml (median)</td>
<td>8.8</td>
<td>31.2</td>
<td>94.2</td>
</tr>
<tr>
<td>Number of subjects out of 8 with consistent response</td>
<td>5</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Priming at baseline</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Priming at 1 hour</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Priming at 6 hours</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The maximum signal for all cytokines was seen at V9 6 hours post challenge.

The graphs of individual subject data allow the signal to be tracked across the challenge week and determine the number of subjects who have a consistent response to repeated challenge.
4.4.4 Effect of fluticasone administration prior to repeated nasal allergen challenge on nasal cytokine levels

Figure 4-8 Effect of fluticasone on IL-4 response to nasal allergen challenge. Data shown as median and interquartile range. There was no IL-4 response to nasal challenge at baseline which makes assessment of priming difficult. There was an increase in levels of IL-4 in nasal lavage post challenge at V9, but this was not statistically significant.
Figure 4-9 Effect of fluticasone on IL-5 response to nasal allergen challenge. Data shown as median and interquartile range. There was no response to nasal challenge at baseline, which makes assessment of priming difficult. There was not an increase in levels of IL-5 in nasal lavage at V9.
Figure 4-10 Effect of fluticasone on IL-13 response to nasal allergen challenge. Data shown as median and interquartile range. There was no response to nasal challenge at baseline which makes assessment of priming difficult. There was an increase in IL-13 levels in nasal lavage at 6 hours post challenge at V9, but this did not reach statistical significance.

Figure 4-11 Effect of nasal fluticasone on eotaxin response to allergen challenge. Data shown as median and interquartile range.
Figure 4-12 Effect of fluticasone on IFNy response to nasal allergen challenge. Data shown as median and interquartile range. There was no response to single or repeated nasal allergen challenge for eotaxin or IFNy.
4.4.5 Effect of repeated placebo challenge on nasal cytokine levels

Repeated nasal challenge with placebo did not cause increases in cytokine levels in nasal lavage. The nasal filter paper data did cause some increases in cytokines in one of the subjects.

Figure 4-13 Effect of repeated nasal challenge with placebo on nasal cytokines in filter paper eluate and nasal lavage (n=2).

Repeated nasal challenge with placebo did not cause increases in cytokine levels in nasal lavage. The nasal filter paper data did cause some increases in cytokines in one of the subjects.
4.4.6 Results summary

Nasal allergen challenge caused an increase in nasal symptoms that was maximal at 15 minutes post challenge. This effect was not abolished by the prior administration of fluticasone. Placebo challenge of the nose did not cause nasal symptoms. Nasal allergen challenge caused an increase in nasal eosinophils in nasal lavage. Priming was assessed by using the Wilcoxon matched pairs test to compare responses across the challenge week at comparable time points, comparing response to allergen challenge at visit 2. The baseline did not increase across the challenge week, however at 1 hour post challenge there were significant increases in the % eosinophils in nasal lavage at 1 hour post challenge at visit 5 (p=0.03), visit 7 (p=0.03) and visit 7 (p=0.05) when compared with the response at visit 2. When the subjects returned a week later at visit 9 this priming effect was no longer present (p=0.3). At 6 hours post challenge there was not a significant increase in response when compared with visit 2.

In the fluticasone group there was no increase in the % eosinophils in nasal lavage following initial challenge. However levels of eosinophils were increased in nasal lavage 1 hour post challenge in subsequent challenges despite the pre-administration of fluticasone. Challenging the nose with placebo allergen did not increase the levels of eosinophils in the subjects studied.

When considering detectable levels of cytokines and chemokines it was striking that detectable levels were generally higher in nasal lavage supernatants than in filter paper eluates. This finding was contrary to our previous studies, where filter paper eluate levels were always higher than those in nasal lavage supernatants. Repeated nasal allergen challenge did not cause a significant rise
in IL-4 levels in nasal lavage. There was an increase in IL-4 in nasal lavage consistently across subjects at V9 6 hours post allergen challenge. There was a trend (non statistically significant) for an increase in IL-4 in nasal lavage following repeated nasal allergen challenge in the fluticasone treated group. Nasal challenge with placebo did cause an increase in IL-4 at one challenge that did not fall back to baseline and as such is anomalous data. The filter paper data did not show any consistent patterns of priming with progressively increasing levels of cytokines and chemokines post nasal allergen challenge performed on consecutive days.

There was an increase in IL-5 at 6 hours post challenge at V9, and this was significantly increased when compared to initial challenge at V9. There was no increase in IL-5 in nasal lavage at any of the challenge visits in the fluticasone treated group. Placebo nasal challenge did not cause an increase in IL-5 levels in nasal lavage.

Repeated nasal allergen challenge caused elevated levels of IL-13 in nasal lavage. There was no evidence of priming pre-challenge or 1 hour post challenge. There was evidence of priming at 6 hours at V7 (p=0.01) and V9 (p=0.008). There was an increase in IL-13 in nasal lavage at 6 hours post challenge at V9, but this did not achieve statistical significance. The fact that there was a response seen at 6 hours post challenge at V9 suggests that priming has occurred. There was a trend within individual subjects for levels of IL-13 to be increased during the final challenge. In the placebo group one subject did have increased levels of IL-13 in nasal lavage during the challenge week, but this was not sustained at the following challenge.
In all the groups and for all of the cytokines the filter paper eluate paper did not show any consistent patterns.

Systemic effects of repeated nasal allergen challenge

The following table summarises the changes in blood levels of IgE (total and specific) and IL-13 at baseline and at study completion. Data shown is median and range.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Allergen n=8</th>
<th>Fluticasone n=4</th>
<th>Placebo n=2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgE baseline</td>
<td>191 (21-788)</td>
<td>142 (43-539)</td>
<td>56, 130</td>
</tr>
<tr>
<td>Total IgE follow up</td>
<td>214 (52-857)</td>
<td>343 (42-622)</td>
<td>55, 152</td>
</tr>
<tr>
<td>Timothy grass IgE baseline</td>
<td>35 (6-86)</td>
<td>20 (8-33)</td>
<td>6, 8</td>
</tr>
<tr>
<td>Timothy grass IgE follow up</td>
<td>36 (13-87)</td>
<td>28 (8-100)</td>
<td>7, 5</td>
</tr>
<tr>
<td>Plasma IL-13 baseline</td>
<td>2.9 (0-116)</td>
<td>9 (5-13)</td>
<td>94, 0</td>
</tr>
<tr>
<td>Plasma IL-13 follow up</td>
<td>2.5 (0-116)</td>
<td>9 (0-12)</td>
<td>87, 0</td>
</tr>
</tbody>
</table>

This table shows that plasma levels of IgE and IL-13 were not increased following repeated allergen challenge, and hence systemic inflammation was not detected.

Regression analysis did not yield any link between baseline levels of total, IgE, Timothy grass specific IgE and plasma IL-13 and subsequent response to nasal allergen challenge.

4.5 Discussion

The aim of the development of a clinical model of repeated daily dosing with allergen was to mimic the exposure that occurs naturally in the allergen season. Many animal models of allergen challenge employ this priming, and novel therapeutics are often effective in the context of inhibiting priming reactions. A
therapeutic that inhibits priming in an animal model can then be assessed in a related human model, which is also similar to natural allergen challenge.

The late phase reaction associated with release of IL-4, IL-5 and IL-13 is of particular interest when assessing new drugs. The best model of the allergen exposure that occurs during the hay fever season is the Vienna chamber, but access to these is limited and the model is not generally employed in the context of repeated sampling.

It is possible that repeated allergen dosing could cause tolerance and hence a reduced response over the course of the challenge week. However the doses of allergen used in immunotherapy shows a dose dependent effect and the doses used to achieve clinical benefit are in the range of 15-20μg (379), this is in comparison to the much lower dose of 0.33μg used in this study hence tolerance is unlikely to have occurred.

One of the limitations of this study was that samples were collected up to 6 hours post challenge and then at 24 hours post challenge. There may have been a greater magnitude of response at 8 hours. There was no evidence of increased eosinophils or cytokines prior to allergen challenge on each of the study days suggesting that priming was not due to an increase in inflammatory cells or mediators that were amenable to collection by nasal lavage, it is possible however that there were changes in inflammatory cell numbers in the mucosa or indeed changes in the nasal epithelium cells that lead to the priming effects seen. This could be investigated further by sampling the mucosa using a nasal scraping technique such as the Rhinoprobe®.

Previous studies from other groups have shown that eotaxin levels are increased in nasal lavage samples following single allergen challenge (98), and
following repeated nasal challenge and this effect can be reduced by the
administration of a nasal steroid (373). However, eotaxin levels did not increase
in this model following single or repeated challenge. The levels of IL-4 in nasal
lavage were comparable to the levels presented in the single challenge model
in chapter 3; however the levels of IL-5 and IL-13 found following repeated
challenge were higher. The raw data plots enable responses for individual
subjects to be visualised and demonstrates the contribution of an individual
subject to the pooled data. This is important because these results show that it
is the same individual having a response to challenge across the study rather
than random contributions from numerous subjects. In this study it was found
that responder rates for IL-4, IL-5 and IL-13 were 5, 4 and 6 out of 8 subjects
respectively. This responder rate after repeat dose allergen challenge is higher
than the responder rate following single allergen challenge reported in Chapter
3. If it could be predicted prior to the study the response rate then this would
allow subjects to be selected for the study, however regression analysis has not
shown that levels of IgE or serum IL-13 at screening can predict who is going to
respond. This is an important point for drug development however as carrying
out trials on selected patients as this type of selection of reactive individuals
may make generalisation of such treatment difficult and would have to be
confirmed by larger more inclusive protocols. With the ever expanding field of
pharmacogenetics the future of drug development is tailoring treatments to
individuals.

Subjects were treated with fluticasone as a positive control group. A single dose
of topical nasal steroid has been shown to reduce symptoms at 8 and 24 hours,
with no effect on acute symptoms (101). This is consistent with the findings of
this study. A reduction in the early phase symptoms has been seen following a
week of fluticasone treatment prior to allergen challenge (380). A similar model to the one used in this study where subjects were given allergen challenges for 7 days following 7 days of nasal steroid and, and this was shown to reduce symptoms and inflammatory mediators in nasal lavage (381). This is not directly comparable with the results of this study as subjects did not receive pre-treatment with nasal steroid prior to the challenge week. In addition the subjects in that study did not have an allergen challenge prior to receiving nasal steroids so it is possible that they were non-responders to allergen challenge.

The main problem with the fluticasone group is that there was not any increase in cytokine levels in response to initial challenge which makes it impossible to detect inhibition of response in subsequent challenges. It is possible that by chance the 4 subjects selected at random for the fluticasone treatment were non-responders to allergen challenge, and this again underlines the need for larger sample sizes. Repeated placebo challenge did not cause increases in levels of cytokines in nasal lavage. This is important because the allergen is stabilised in phenol and it is essential to determine that this does not cause a non-specific increase in vascular permeability and hence increase levels of cytokines in nasal lavage by a non-immune mechanism. This was further clarified by the measurement of IFNy in the nasal lavage sample, as this would not usually be released following allergen challenge, but may be increased in generalised capillary leak. However it is important to note that the subjects in the placebo group did have lower levels of IgE and hence may be considered to be less atopic.

One of the main limitations of this exploratory study was the small number of subjects in the fluticasone and placebo groups, but unfortunately this was a
fixed part of the protocol and further funding could not be obtained to increase the sample size.

This study did not show any systemic effects of repeated allergen challenge and determined by blood levels of IgE and IL-13. It has previously been shown that nasal allergen challenge does not increase levels of eosinophils in the blood or sputum (382). Safety of subjects participating in research is extremely important and tolerability of a model is important. There were no serious adverse events reported during this study, there were several adverse events of subjects reporting frontal headaches during the challenge week that were similar in nature to the headaches they suffer during the hay fever season. These headaches were treated with paracetamol and no subject withdrew from the study as a result.

The detectable filter paper eluate levels of cytokines were consistently low in this study, and showed no relation to the levels found in the nasal lavage. There was an increase in nasal symptoms, eosinophils and cytokines in nasal lavage following challenge suggesting that there was not a problem with the allergen or its delivery device. The Whatman 42 filter paper is designed as a cellular filter and is not designed for protein work. However Whatman 42 is the paper used in the original description of the method (329).

In preliminary experiments, an absorptive synthetic matrix that is designed for protein elution use was assessed by members of the Clinical Studies Unit (results presented in Appendix 2). Accuwik Ultra® (Pall Gellman) was used in spiking experiments and shown to have improved protein recovery compared to Whatman's FILT P. Therefore in any subsequent clinical studies the Accuwik® paper could be used, although batch validation is also recommended for this
product. Also in the appendix is the results of a study subsequently performed using the Accuwik® paper in a nasal allergen challenge study that shows increased levels of IL-13 following nasal allergen challenge with an increased dose of Timothy grass. In general, the levels in lavage are lower due to dilutional effects; hence it is preferable to use an absorptive matrix to obtain a bigger signal.

4.6 Conclusions

This repeat dose NAC model does cause significant priming of the IL-5 and IL-13 late phase response in nasal lavage, with a trend for increased symptoms and eosinophils.

Repeat NAC is feasible for recruitment and the protocol is safe and well tolerated by subjects.

Repeated NAC could be used as a model for the assessment of new drugs, especially those therapies that target IL-4, IL-5 and IL-13.

4.7 Future study

1. It would be beneficial to determine subjects' responsiveness to allergen challenge at screening prior to inclusion in the study, with documentation of serial nasal cytokine and chemokine responses.

2. One should only employ Whatman's filter paper if it has appropriate elution characteristics for a particular batch demonstrated by spiking with commercial standards and nasal samples. Otherwise there is the need to identify and validate novel absorptive matrices with reproducible and efficient elution characteristics.
3. The dose of allergen employed should be characterised, since greater doses may cause higher detectable cytokine responses.
5 Cigarette smoke nasal challenge in patients with COPD

5.1 Abstract

Introduction: Cigarette smoke is the commonest cause of COPD, and has been shown to have inflammatory effects in animal models and in in vitro human models. For ethical reasons an in vivo human cigarette smoke challenge model has not been developed. Nasal challenge has been used effectively to show the inflammatory changes in allergic disease and could potentially be used to demonstrate the inflammatory effects of smoking.

Aims: The aim of this exploratory study was to assess whether there will be an inflammatory response (detectable as changes in nasal inflammatory mediators) following exhalation of cigarette smoke through the nose.

Methods: A total of 8 smokers with COPD and 8 smokers with no evidence of lung disease as determined by symptoms and spirometry were recruited. They abstained from smoking for 12 hours and then smoked a cigarette with the smoke being exhaled through the nose. Nasal filter paper and nasal lavage samples were collected prior to challenge, and at time intervals up to 8 hours following challenge for cytokine/chemokine and cell analysis respectively. Samples of blood were also collected for cytokine/chemokine analysis of plasma.

Results: There was a trend for cytokine/chemokine levels to be higher in nasal filter paper samples and plasma samples in the subjects with COPD. In nasal filter paper samples there was a statistically significant difference for IL-8 and IL-12p70 at baseline after washing. In nasal lavage samples cytokine and chemokine levels were similar for both groups; however there was an increase
in MCP-1 8 hours following challenge. There was a pattern of increased neutrophils in nasal lavage following challenge in 3 out of 8 COPD subjects. In plasma samples there was a statistically significant difference for IFN-γ and MIP-1α. None of the cytokines or chemokines measured in nasal filter paper eluate samples or plasma were increased in response to nasal cigarette challenge, but based on the NAC studies the FILT P is likely to have been inadequate for detection of inflammatory mediators in nasal secretions.

**Conclusions** This pilot study suggests that further development of this model is justified. It should be repeated with more subjects who are more carefully screened to exclude the presence of respiratory disease. Attention should also be paid to length of abstinence from smoking prior to challenge and also to continued collection of samples beyond 8 hours. The nasal filter paper samples should be collected using a SAM designed for protein absorption and release such as the Accuwik® paper.

**5.2 Introduction**

**5.2.1 The role of smoking in the pathogenesis of COPD**

Cigarette smoking is by far the commonest cause of COPD worldwide, accounting for more than 95% of cases in industrialised countries. Usually there is a smoking history of greater than 20 pack years. Smoking causes both mucus hypersecretion and progressive airflow obstruction. Smoking cessation decreases respiratory symptoms and slows the rate of decline of lung function (383-387). Passive smoking is associated with the development of COPD (388-390) and smoking during pregnancy may be a risk factor for the development of COPD as this may affect foetal lung growth (391). As discussed in Chapter 1,
there is considerable evidence from *in vitro* and *in vivo* models that the development of COPD as a result of cigarette smoking is biologically plausible.

### 5.2.2 Nasal disease in patients with COPD

Whilst the name COPD suggests a disease affecting the lungs, it has been apparent that there are effects on the body distant from the lungs (392). The nasal mucosa is continuous with the respiratory mucosa and also is exposed to cigarette smoke. It is therefore not surprising that nasal disease is present in smokers with COPD. A study of patients with COPD has shown that raised nasal levels of IL-8 are seen in patients with COPD and this correlates with levels of IL-8 present in sputum and nasal wash leukocyte concentration (393). A study comparing nasal and bronchial biopsies in smokers with and without COPD has that nasal and bronchial inflammation coexists in smokers and is characterised by infiltration of CD8 T lymphocytes. In smokers with COPD this is associated with an increased number of neutrophils in both nasal and bronchial biopsy specimens (249). Markers of inflammation have been shown to be increased in nasal wash samples at the time of exacerbation suggesting that COPD exacerbation is associated with pan-airway inflammation (244). In a very interesting study patients with COPD showed an increase in markers of neutrophil but not eosinophil inflammation following challenge with histamine and this was greatest in subjects who complained of nasal symptoms prior to challenge (250). The same study also showed that COPD patients with nasal symptoms had increased mucinous secretory hyperresponsiveness.

### 5.2.3 Nasal challenge models

The nasal epithelium is histologically similar to and in continuation with the respiratory epithelium and has the advantage of being easily accessible.
Therefore the nose has been used to study the effects of various stimuli. As discussed previously in this Thesis the nose can be used to study responses to allergen challenge. Lipopolysaccharide nasal challenge has been shown to increase GM-CSF in nasal lavage fluid four hours after challenge in atopic and non-atopic subjects (394). LPS has been shown to cause a release of IL-6 into nasal lavage 6 hours following challenge. IL-1β, IL-6, IL-8 and TNFα, and neutrophils are all increased in nasal lavage fluid at 6 hours post nasal challenge with lipopolysaccharide. Ozone is an environmental pollutant that has oxidant properties, and has been shown to have inflammatory properties in human airways(395;396). Exposure to ozone causes a central regulator of epithelial function, epidermal growth factor receptor, to be increased in nasal biopsy specimens following exposure to ozone (397).

5.2.4 Cigarette smoke challenges

It is possible to generate cigarette smoke extracts by bubbling cigarette smoke through liquid which can then be added to cell culture lines or other tissue preparations and then measure the inflammatory response. It is also possible to investigate the effects of single components of cigarette smoke such as nicotine or acetaldehyde, although this method has limitations due to the complex chemical nature of cigarette smoke. It has been suggested that some of the effects seen in cigarette challenge models is due to the effect of lipopolysaccharide present in the tobacco, however it has been shown that cigarette conditioned medium does not contain significant amounts of lipopolysaccharide using the Limulus assay (398). Similarly in vivo animal models of cigarette smoking can be divided into those that look at the effect of cigarette smoke or those that look at specific
components of cigarette smoke. Animals can be exposed to cigarette smoke by using smoking machines that deliver a constant rate of smoke to their cages, and the design can be modified to create models of mainstream or sidestream passive smoking. These models have been used to show the effects of exposure to cigarette smoke in animals (399;400). It has been shown that inflammatory changes similar to those seen in COPD can be induced in mice by intranasal application of cigarette conditioned medium (401).

In humans there are considerable ethical issues when planning a cigarette smoke challenge model. It is not ethical to expose non-smokers to cigarette smoke. Similarly it is not ethical to expose smokers to individual components of cigarette smoke as the exact levels reached in the tissues cannot be accurately known and hence subjects may be exposed to levels much higher than those reached during normal smoking and hence suffer form greater toxicity. In a human smoking model were samples were collected following smoking there was and increase in sputum neutrophils and lymphocytes and greater II-8 release from stimulated blood cells (402). This model allows subjects to be exposed to levels of cigarette smoke associated with normal smoking, and also allows multiple samples to be taken following the exposure.

5.2.5 Aims

The aim was to develop a model for the testing of new anti-inflammatory drugs for the treatment of COPD. Patients with COPD often have reduced respiratory reserve so the aim was to develop a model that avoided the need for invasive procedures such as bronchoscopy. It was hoped that using a challenge model would increase the inflammatory signal to increase the power of any future studies with new therapeutic entities.
5.3 Methods

5.3.1 Subject selection

This study received approval from a local ethics committee. Subjects were recruited from the database at the Clinical Studies Unit and by advertisements placed in local newspapers. Subjects underwent a full medical and smoking history, physical examination and lung function as detailed in section 2.1.1-2.1.3. Subjects in the COPD group were given a subject number from 101-108, and the “healthy” group were given a number from 201-208.

5.3.2 Nasal sample collection

Nasal lavage and filter paper samples were collected as detailed in section 2.1.5 and 2.1.6. Briefly nasal lavage samples were obtained by instilling 5 mls of PBS into the left nostril with the subjects’ neck flexed. The PBS was flushed slowly in and out of the nose 20 times. The lavage sample was then centrifuged and a cell pellet obtained for cell counting and the supernatant retained for cytokine and chemokine analysis. The right nostril had 2 pieces of filter paper inserted for 2 minutes. These were then placed into Luminex® assay buffer and the eluate retained for cytokine and chemokine analysis.

5.3.3 Plasma collection

Blood was collected for cytokine analysis as detailed in section 2.1.8.

5.3.4 Measurement of cytokines and chemokines

Nasal lavage supernatant, nasal filter paper eluate and plasma were analysed using the Luminex® multiplex analyser. All samples were stored at -80°C from time of collection until analysis.
5.4 Results

5.4.1 Subject clinical data

There were 8 subjects with COPD and 8 subjects with no clinical evidence of lung disease who continue to smoke at least 5 cigarettes a day were recruited for this study. Their clinical details are summarised in Table 5.1. Subjects were excluded if they had allergic rhinitis or within two weeks of an upper respiratory tract infection.

Table 5.1 Subject demographics

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Healthy smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean, range)</td>
<td>64 (48-75)</td>
<td>55 (42-71)</td>
</tr>
<tr>
<td>Gender (% Female)</td>
<td>50</td>
<td>37.5</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>All Caucasian</td>
<td>All Caucasian</td>
</tr>
<tr>
<td>Smoking history: pack years (mean, range)</td>
<td>43.5 (17.5-67)</td>
<td>35.91 (16.8-82.5)</td>
</tr>
<tr>
<td>Current smoking: number of cigarettes/day (mean, range)</td>
<td>17.75 (12-20)</td>
<td>19.94 (7.5-35)</td>
</tr>
<tr>
<td>FER pre bronchodilator (mean, range)</td>
<td>49.13 (27-63)</td>
<td>77.50 (73-84)</td>
</tr>
<tr>
<td>FEV1: % predicted (mean, range)</td>
<td>58.5 (26-78)</td>
<td>99 (94-105)</td>
</tr>
</tbody>
</table>

5.4.2 Nasal lavage cytology

Nasal lavage cytology results are expressed as % neutrophils per ml of lavage fluid. This is calculated by multiplying total cell count from a Neubauer chamber by % neutrophils on a cytospin stained with Diffquick®. This was then corrected for the amount of lavage recovered from the patient.
Figure 5.1 Neutrophils/ml of nasal lavage fluid following challenge with one cigarette in COPD and healthy smokers.

Figure 5.2 shows the results for COPD and healthy subjects following nasal challenge with one cigarette. It can be seen that healthy smokers had low levels of neutrophils at baseline, whereas three of the COPD subjects had raised neutrophils at baseline. One subject in the COPD had remarkably high levels of neutrophils at baseline. It is possible that the pre-lavage was not performed adequately. Three subjects in the COPD group and one subject in the healthy group showed some increase in neutrophil levels in nasal lavage following nasal cigarette challenge.
5.4.3 Nasal filter paper cytokines and chemokines

**IL-1a**

- **COPD**
  - Median + IQR
- **Healthy**
  - Median + IQR

**IL-1b**

- **COPD**
  - Median + IQR
- **Healthy**
  - Median + IQR

**IL-2**

- **COPD**
  - Median + IQR
- **Healthy**
  - Median + IQR

**IL-3**

- **COPD**
  - Median + IQR
- **Healthy**
  - Median + IQR
Figure 6. Comparison of cytokine/protein levels before and after challenge in COPD and healthy subjects. Following nasal antigen challenge, baseline levels were compared using a Student’s t-test. There were significant differences between COPD and healthy subjects at baseline (IL-6 p=0.01, IL-10 p=0.03).
Figure 5-2 Levels of cytokines/chemokines in nasal filter paper eluates from smokers with COPD and healthy smokers following nasal cigarette smoke challenge. Data shown as median and interquartile range. Baseline levels were compared using a Mann-Whitney test. There were significant differences between COPD and healthy subjects at baseline for IL-8 (p=0.01) and IL-12p70 (p=0.02).
5.4.4 Nasal lavage cytokines and chemokines

Figure 5-3 Levels of cytokines/chemokines in nasal lavage from smokers with COPD and healthy smokers following nasal cigarette smoke challenge. Data shown as median and interquartile range. Baseline levels for the 2 groups were compared using a Mann-Whitney test, and showed no differences. Responses at 8 hours compared with baseline in each group were compared with the Wilcoxon signed rank test. There was an increase in MCP-1 following challenge in both COPD and healthy subjects.
5.4.5 Plasma cytokines and chemokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>COPD</th>
<th>Healthy</th>
<th>Median + IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
</tr>
<tr>
<td>IL-5</td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
</tr>
<tr>
<td>IL-6</td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
<td><img src="image9.png" alt="Graph" /></td>
</tr>
<tr>
<td>IL-7</td>
<td><img src="image10.png" alt="Graph" /></td>
<td><img src="image11.png" alt="Graph" /></td>
<td><img src="image12.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
COPD Healthy Median + IQR

IL-15

GM-CSF

INF-γ

TNF-α

Hours post challenge
Figure 5-4 Levels of cytokines/chemokines in plasma samples from smokers with COPD and healthy smokers following nasal cigarette smoke challenge. Data shown as median and interquartile range. Baseline levels were compared using a Mann-Whitney test. There were significant differences at baseline between COPD and healthy groups for IFN-γ (p=0.01) and MIP-1α (p=0.05).
5.4.6 Results summary

In the analysis if the filter paper eluates there are a trend for levels of cytokines and chemokines to be elevated at baseline in the COPD subjects when compared to the healthy controls. However this only reaches statistical significance for IL-8 (p=0.01) and IL-12p70 (p=0.02). It would appear that none of the cytokines or chemokines increases in nasal filter paper samples in response to nasal cigarette smoke challenge.

For the nasal lavage data baseline levels of cytokines and chemokines were similar for each of the groups. There was a response following nasal cigarette challenge for MCP-1 in both groups. This reached statistical significance at 8 hours for both COPD and healthy subjects (p=0.02)

There is a trend for increased plasma levels in COPD smokers for IL-6, IL-7, IL-12p70, IL-13, GM-CSF, IFN-γ, eotaxin and MIP-1α. There is a significant difference between COPD smokers and healthy smokers at baseline for IFN-γ (p=0.01) and MIP-1α (p=0.05). None of the cytokines or chemokines measured in plasma were increased in response to nasal cigarette smoke challenge.

5.5 Discussion

This was a pilot study to investigate if a signal of inflammation could be detected following nasal cigarette smoke exposure as a potential model for the testing of new drugs for COPD.

Traditionally sputum leukocyte counts are calculated by assessing leukocyte numbers using a Neubauer chamber and then performing a leukocyte differential on a cytospin preparation. In sputum preparations the distinction between squamous epithelial cells and leukocytes in a Neubauer chamber is
clear. However in nasal lavage preparations there are large numbers of respiratory epithelial cells in addition to squamous epithelial cells. On this unstained preparation it is very difficult to distinguish between a leukocyte and a small respiratory epithelial cell, relying on granularity differences can be misleading as macrophages may have limited granularity. Similarly on a cytospin preparation there are epithelial cells of intermediate size that are difficult to classify into squamous or respiratory epithelial types. Therefore the most robust way to interpret the cytology of nasal lavage in this population is to determine the neutrophil % of total cells on a cytospin preparation stained with Diffquick® and then multiply this by the total cell count obtained on a Neubauer. This can then be corrected for the number of mls of lavage recovered from the subject. This is in contrast to nasal lavage cell counting following an allergen challenge where it is easier to determine the leukocyte sub-types, and the eosinophil is usually absent at baseline and rises to approximately 30% of the leukocyte total. There also appears to be less contamination of the post-allergen challenge slides with indeterminate epithelial cells.

Modification of the staining methodology may lead to more accurate expression of the neutrophil count. Use of the May-Giemsa-Grunwald stain may differentiate the leukocyte subtypes more clearly. However the optimal method would be immunohistochemistry, using CD68 to identify macrophages and cytokeratin 10 to distinguish the squamous cells. Vimentin would not be suitable as it has been shown to be present on cytokeratin positive cells in addition to the ciliated or goblet type cells (403).

This pilot data shows that there is some merit in repeating this study with a larger number of subjects. It would also be interesting to repeat lavages more
frequently in the first hour, at 10, 20 and 30 minutes. This study could incorporate a control group who did not smoke a cigarette to determine if repeated washing had an effect of nasal lavage cell counts and mediator levels.

It is interesting to note that even with the small numbers present in this study there were differences in nasal filter paper and plasma cytokines and chemokines between smokers with COPD and healthy smokers. There was the suggestion that some of the mediators were different but these did not reach statistical significance, which may be a reflection of the small number of subjects. This study did not have a control group of non-smokers and this would be a useful group to compare with the smokers, but would not be possible to study for ethical reasons. There is a problem interpreting the nasal filter paper data, as in the previous chapter we have shown that Whatman’s 42 paper may cause degradation of cytokines and chemokines. Therefore any future studies should be carried out with a paper such as the Accuwik Ultra® that has had its elution characteristics established using commercially available cytokine and chemokine standards.

There was not a stimulatory response to nasal cigarette smoke in nasal samples or plasma samples, with the exception of MCP-1 in nasal lavage samples. The filter paper used in this study was of the same batch as was used in the repeat dose allergen model and hence the results should be reviewed with caution. However the nasal lavage data should give an accurate representation of the changes occurring in the nose following challenge. There are two possible explanations for this. It is likely that abstinence for 12 hours followed by a single nasal cigarette is not a potent enough stimulus to detect a change, and levels may not have fallen from the cigarette the previous evening.
It is also possible that 8 hours is an insufficient time period for inflammation to develop, and that an inflammatory response may have been seen if samples were taken at a later time point. COPD develops over a prolonged period of time with repeated cigarette exposure and this model does not replicate the pathogenesis of COPD.

It is also possible that the so-called "healthy" smokers actually do have an inflammatory process that was not detected in the screening for this study. Subjects were defined as healthy in this study if they were free from respiratory symptoms and had normal FEV$_1$ and FVC on spirometry. In future studies it might be beneficial to characterise the healthy subjects further by performing full lung function testing to assess MEF25-75 to assess any small airways disease and transfer factor to suggest any early emphysema. A high resolution CT scan of the thorax could also indicate the presence of emphysema or another smoking related lung disease such as respiratory bronchiolitis- interstitial lung disease.

5.6 Conclusions

This model of nasal cigarette challenge has the potential to be used to characterise the inflammatory changes that occur in smoking related lung disease and also to be used for the evaluation of new anti-inflammatory therapies for the treatment of COPD.

5.7 Future study

Use an increased number of subjects in each group and use SAM to obtain nasal samples.
Characterise subjects further at screening with CT scans and full lung function, to ensure that “healthy” group are free of any respiratory disease.

Collect samples at time points beyond 8 hours.

Refine laboratory techniques of nasal lavage cytology and also determine the elution characteristics of an alternative matrix for the collection of nasal filter paper samples.
The effects of cigarette smoke conditioned medium (CSCM) and oxidants (menadione and xanthine/xanthine oxidase) on neutrophil CD11b upregulation in human whole blood.

6.1 Abstract

Introduction: Cigarette smoke is the main cause of COPD, and contributes to cardiovascular disease and malignancies. Cigarette smoke is a rich source of oxidants, and further oxidant stress is caused by accumulation of inflammatory cells. Cigarette smoke conditioned medium (CSCM) has been shown to stimulate the release of inflammatory mediators from human cells \textit{in vitro}, and is a source of oxidants, tar, lipopolysaccharide and other molecules. As a more specific oxidant stimulus, menadione crosses cell membranes and leads to the generation of reactive oxygen species intracellularly, whereas the xanthine/xanthine oxidase system leads to the extracellular generation of reactive oxygen species.

Aims: To develop a short term stimulation model of whole blood with CSCM and oxidants through flow cytometric quantification of neutrophil CD11b upregulation.

Methods: Whole blood short term stimulation systems were developed with (i) CSCM (ii) menadione and (iii) xanthine/xanthine oxidase. Following stimulation, cells were fixed prior to labelling, and CD11b upregulation on leukocytes was measured using flow cytometry. In experiments designed to inhibit the effect of oxidants, reduced glutathione and N-acetylcysteine were used as anti-oxidants.

Results: Fresh CSCM and oxidants caused human neutrophil levels of CD11b to become upregulated in a dose dependent manner. The effect of CSCM could
not be inhibited by anti-oxidants, but there was loss of CSCM stimulation on storage on the bench. There was no significant difference in the CD11b response to CSCM stimulation of neutrophils between “healthy” smokers and COPD smokers. In contrast, menadione caused a dose dependent increase in CD11b expression on neutrophils that could be inhibited by the presence of reduced glutathione. Xanthine/ xanthine oxidase required plasma to be removed from whole blood before a dose dependent increase in CD11b on neutrophils could be demonstrated, and these effects could again be inhibited by glutathione.

Conclusions: CSCM, menadione and xanthine/xanthine oxidase all cause neutrophil CD11b upregulation. These whole blood models have the potential for use pre clinically to screen in vitro new anti-oxidants and anti-inflammatory treatments for COPD.

6.2 Introduction

6.2.1 Role of oxidative stress in COPD

There is considerable evidence that oxidative stress in increased in patients with COPD (128;130-132). This arises because of the reactive oxygen species (ROS) present in cigarette smoke and also the inflammatory processes triggered by inhaling cigarette smoke. Therapies targeted at oxidant stress should offer an anti-inflammatory treatment for COPD and developing a preclinical human model to test potential new therapies could lead to enhanced development in this area. Human whole blood assays can be used pre clinically (in vitro), but can also be employed to assess pharmacodynamics in clinical pharmacology studies during phase I/II clinical studies ex vivo. It has been shown that the pharmacodynamics of a specific p38 mitogen-activated protein
kinase inhibitor can be assessed by measuring cytokine production from ex-vivo stimulated polymorphonuclear blood cells in a phase one clinical trial (404). Pharmaceutical companies are keen to shorten timelines for drug development and an ex-vivo model for testing new compounds in a model of oxidant stress could reduce the time it takes potential new therapies to proceed to clinical testing.

6.2.2 Effects of CSCM on biological systems

Cigarette smoke medium is produced by passing the smoke from a burning cigarette though cell medium and then applied to the target cells. Cigarette smoke can stimulate human cells in vitro to release inflammatory mediators (189). It has been shown that CSCM is toxic to an alveolar type II cell-derived line, and this toxicity could be inhibited by aldehyde dehydrogenase and N-acetyl cysteine (405).

CSCM triggers stress responses in cultured fibroblasts by the induction of haem oxygenase and a significant decrease in intracellular glutathione (406). When c-fos is used as an indicator of cellular response against oxidative stress, it's expression in fibroblast cells can be inhibited by superoxide dismutase and catalyse or the NO-scavenger oxyhaemoglobin (407). The mechanism by which cigarette smoke causes these changes in vivo has yet to be well characterised. CSCM induces IL-8 release and expression in human airway smooth muscle cells, without effect on eotaxin or RANTES, while TNFα enhances CSCM induced IL-8 release and expression. The effects of CSCM on IL-8 release can be inhibited by glutathione and associated with the induction of the oxidant sensing protein, haem oxygenase-I (408). This effect on IL-8 without effect on
eotaxin or RANTES may explain why the inflammation in COPD is predominantly neutrophilic.

CSCM has been shown to stimulate production of IL-8 in human macrophages, and this can be inhibited by the addition of N-acetyl cysteine and this is mediated by AP-1 but not NF-κB (238). The role of NF-κB in CSCM mediated cytokine release is controversial as it has been shown that smoke can activate (409), inhibit the activation (410), or not affect NF-κB (411). Similarly contradictory results have been found with regard to AP-1 (410;412;413). Some of these differences may be explained by different methods of preparation, and different concentrations of CSCM being used, as there is no universally agreed standardisation method.

6.2.3 Oxidants and Toll-Like Receptors (TLRs)

The recent discovery of the TLR family has increased our understanding of the innate immune system, especially the way that the TLRs act as sensors for infection. TLRs are pattern recognition receptors (PRRs) for "pathogen associated molecular patterns" (PAMPs), acting as surveillance receptors for microbial products and oxidants (414;415). Gram positive bacterial and fungal lipopeptides bind to TLR2, which forms heterodimers with either TLR1 or TLR6. In contrast, Gram negative lipopolysaccharide (LPS) binds to TLR4 in association with CD14 (239). It has recently been shown that oxidants, possibly via TLR2, prime monocytes from smokers for challenge via bacteria and TLRs, causing enhanced inflammatory responses (242). Macrophage phenotype and activation are regulated by cytokines that use the Jak-STAT signalling pathway, TLRs and Immunoreceptor Tyrosine-based Activation Motifs (ITAMS). There is
cross talk between the signalling pathways, and dysregulation of this cross talk may contribute to the pathogenesis of chronic inflammatory diseases (416).

6.2.4 Effects of menadione and xanthine/xanthine oxidase on biological systems

Menadione (Vitamin K₃) has the molecular formula 2-methyl-1, 4-naphtoquinone. It generates superoxide through auto oxidation of one electron-reduced menadione or inhibition of the mitochondrial respiratory chain, which also generates H₂O₂. Menadione also causes superoxide generation through non-enzymatic interaction with thiols such as reduced glutathione; it also inhibits glutathione reductase and causes depletion of reduced glutathione (417). It is important to note that these processed occur intracellularly, the menadione must cross the cell membrane before it exerts it oxidant effects. The effects of vitamin K analogues on human neutrophil function have been evaluated (418). Menadione inhibits neutrophil spreading, locomotion, phagocytosis, degranulation of lysosomal constituents, bactericidal activity, superoxide generation, hydrogen peroxide production and microtubule assembly. A concentration of 10⁻⁵ M produced 50% inhibition of these functions. Xanthine is a precursor of uric acid, and is converted to uric acid by xanthine oxidase. This reaction leads to the formation of superoxide ions, and the addition of xanthine and xanthine oxidase to blood will lead to superoxide generation in the extracellular space and hence could be utilised as a model of oxidative stress. This model has previously been used to determine effect of oxygen radicals on receptor-dependent nitric oxide production by coronary endothelium (419).
6.2.5 Use of flow cytometry in the study of leukocytes

Flow cytometry allows the physical characteristics of a single cell to be determined as it passes through a chamber where lasers are shone on it. The use of labelled anti-bodies also allows the immunological characteristics of the cell to be determined. In the case of whole blood it is possible to identify the leukocyte population using the nuclear dye LDS-751. Within the leukocyte population it is possible to discriminate the granulocytes (neutrophils/eosinophils) from other cells based on their physical (forward scatter and side scatter) properties. Figure 6.1 shows a dot-plot of whole blood, the threshold of the cytometer can then be adjusted so that the RBCs and platelets are removed from the analysis.

![Diagram of dot-plot](image)

Figure 6.1 Dot-plot of whole blood stained with LDS-751 which is brightest in the FL-3 channel plotted against the side scatter characteristics. R1= RBCs and platelets, R2= granulocytes, R3= lymphocytes, R4= monocytes.

A gate can then be placed around R2 or R4 depending if neutrophils or monocytes are to be studied. The use of anti-CD14 for monocytes and anti-CD16 for neutrophils allows these cells to be distinguished. A plot can be made
of CD11b against CD16 to determine CD11b levels on neutrophils, since low levels of CD16 are present on eosinophils, and these can be gated off as shown in fig 7.2 below. The mean fluorescence intensity of the FL-1 channel where FITC bound to CD11b is detected indicates the level of CD11b expressed on cells. The size of cells can also be determined by analysing their forward scatter characteristics, while the granularity of cells influences their side scatter.

Figure 6-2 Dot-plot of CD16-PE in the FL-2 channel against CD11b-FITC in FL-1 channel. There are 2 distinct populations, the population on the right with higher levels of CD16 are the neutrophils and those on the left are the eosinophils. A gate can be placed around the neutrophils and the geometric mean of the CD11b level determined.

When performing short term stimulation assays to cause shape change, a measure of chemokinesis and chemotaxis, the change in shape is transient and very sensitive. Hence, Shannon Bryan and colleagues developed a system where a gentle fixation was performed immediately after stimulation, and prior to cell lysis and flow cytometry (420).

An analogous situation is found for CD11b, where levels on leukocytes can rapidly fluctuate with venepuncture, cooling, incubation and storage. A normal flow cytometry cell surface marker labelling protocol has the fixation step
following the antibody labelling step because it is performed on unstimulated cells with stable levels of cell surface markers. Grant Nicholson and colleagues introduced a light fixation step immediately after short term stimulation, prior to antibody binding (see figure). Grant Nicholson and colleagues developed this method to permit detection of CD11b on the surface of leukocytes in response to stimulation with chemokines (421), and in this chapter this method is adapted to oxidant stimulation of CD11b upregulation.

Figure 6-3 Protocol for whole blood stimulation for CD11b labelling

6.2.6 CD11b upregulation

CD11b (Mac-1, αMβ2) is found both in preformed cytoplasmic granules and on the cell surface of leukocytes, and is combined with CD18 to form one of the β2-integrins. In combination with CD18, CD11b acts as a leukocyte adhesion molecule by binding to intercellular adhesion molecule- (ICAM-) 1 and a complement receptor (CR3). The exact mechanism of rapid CD11b
upregulation on the cell surface remains to be clarified, but involves recruitment of preformed CD11b from granules as well as membrane externalisation. It has been shown that G-protein activation is necessary for CD11b upregulation but the process is not simply a by-product of granule exocytosis (422). It seems that avidity changes due to re-configuration at the cell surface are also important in activation of CD11b (423).

It has also been shown that intracellular calcium, tyrosine kinase and MAP kinase are all involved in β2-integrin expression. Celecoxib (specific COX2 inhibitor) leads to superoxide anion generation within neutrophils and this in turn causes β2-integrin upregulation which can be inhibited by superoxide dismutase (424). Exogenously applied H2O2 and TNFα triggers CD11b activation and can be inhibited by tyrosine kinase inhibitors, and the activation of the tyrosine kinase receptors involves oxidoreduction reactions as the activation can be inhibited by the free radical scavengers N-acetylcysteine, glutathione, and iron (425). It has also been shown that TNF-induced β2 integrin activation involves redox-regulated activation of p38 MAPK (426).

CD11b is upregulated in inflammatory states and disease. Patients with tuberculosis have higher expression of CD11b than healthy controls, and following LPS administration to healthy volunteers there is increased leukocyte CD11b upregulation (427). It has been shown that smokers with COPD have lower maximal shape change for neutrophils in response to IL-8 stimulation in comparison to healthy smokers and a CXCR2 antagonist was found to inhibit neutrophil CD11b upregulation and shape change in COPD patients (421).
6.2.7 Aims

Our aim was to develop a system of short term stimulation of human whole blood with CSCM, menadione and xanthine/xanthine oxidase. We hoped to identify effects of these known oxidant stimuli in terms of CD11b upregulation on neutrophils and monocytes. We wondered whether CD11b levels and responses may differ in COPD patients and smokers, and whether antioxidants and other drugs (such as corticosteroids) could inhibit CD11b upregulation. Finally, it is possible that oxidants could cause effects via TLR2, and prime for responses through other TLRs. Oxidants could thus act on particular cell signalling pathways, and might cause production of cytokines such as IL-8.

6.3 Subjects, Materials and Methods

All subjects gave full informed consent for a blood sample and ethical approval was obtained from the independent Ethics Committee of the Royal Brompton, Harefield and NHLI Ethics Committee.

Healthy volunteers were non-smoking adults, who had not smoked in the past, and without any significant infective/inflammatory/autoimmune/metabolic/neoplastic disease in their past or current medical history.

Subjects with COPD were all former heavy cigarette smokers and defined according to GOLD guidelines (2005) and classified for severity according to post-bronchodilator FEV₁.
Table 6-1: Demographics of subjects used to study CSCM upregulation of neutrophil CD11b

<table>
<thead>
<tr>
<th></th>
<th>COPD n=8</th>
<th>Healthy smokers n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean, range)</td>
<td>64 (48-75)</td>
<td>55 (42-71)</td>
</tr>
<tr>
<td>Gender (% Female)</td>
<td>50</td>
<td>37.5</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>All Caucasian</td>
<td>All Caucasian</td>
</tr>
<tr>
<td>Smoking history: pack years (mean, range)</td>
<td>43.5 (17.5-67)</td>
<td>35.91 (16.8-82.5)</td>
</tr>
<tr>
<td>Current smoking: number of cigarettes/day (mean, range)</td>
<td>17.75 (12-20)</td>
<td>19.94 (7.5-35)</td>
</tr>
<tr>
<td>FER pre bronchodilator (mean, range)</td>
<td>49.13 (27-63)</td>
<td>77.50 (73-84)</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;: % (mean, range)</td>
<td>58.5 (26-78)</td>
<td>99 (94-105)</td>
</tr>
</tbody>
</table>

6.3.1 Preparation of CSCM and oxidant stimuli

CSCM was made by bubbling the smoke of 6 cigarettes through PBS using the apparatus shown in figure 6-4, which was installed in an operating fume cupboard. After the CSCM was made and filtered through a 0.2μM filter it was diluted with PBS on the basis of optical density measured on a spectrophotometer at a fixed wavelength of 320nM (428;429). An optical density of 0.15 was taken to represent a CSCM concentration of 1 Arbitrary Unit (AU) based on previously published work (398). Due to corrosive effects of cigarette smoke constituents on the equipment, I used fresh equipment to prepare each batch of CSCM. Fresh CSCM was prepared on the day of each experiment, stored on ice, and employed within 30 minutes.
The following reagents were used. Menadione (Sigma-Aldrich, MO cat 67900), xanthine (Sigma, St Louis, MO cat X0626) and xanthine oxidase (Sigma, St Louis, MI cat X-2252).

Menadione was initially solubilised in DMSO to make a 0.1M solution and then serially diluted in RPMI or PBS. Xanthine was initially solubilised in NaOH to make a 0.25M solution and then serially diluted in PBS. Microbial xanthine oxidase was diluted in PBS. These solutions were stored at -20°C for a maximum of 6 weeks and serially diluted immediately prior to use.

6.3.2 Preparation of anti-oxidants

Reduced glutathione (Sigma-Aldrich, St Louis, MO, Cat G4251) was dissolved in PBS to give the required concentrations. N-acetylcysteine (-Aldrich, St Louis, MO, Cat A7250) was dissolved in modified RPMI with HEPES buffer to maintain a neutral pH. Fresh solutions were made immediately prior to use.
6.3.3 Venepuncture technique to obtain blood samples

Blood was gently taken by hand using a syringe and a 21G "butterfly" needle into sterile EDTA. Vacutainers were not used since the rapid withdrawal of blood causes CD11b upregulation. Blood was incubated at 37°C for up to 10 min before use. Cooling and agitation were observed to cause CD11b upregulation.

6.3.4 Washing of blood

In the experiments using washed cells 3mls whole blood anti-coagulated with EDTA was made up to 12 mls using phosphate buffered saline (PBS) at 37°C and spun at 400g for 10 minutes. The supernatant was removed taking care not to disrupt the buffy coat; the cell suspension was then restored to 3mls using PBS. The washed blood was then left to rest for 60 minutes in a water bath at 37°C. A paired sample of whole blood was also placed in the water bath for 60 minutes to act as a control.

6.3.5 Stimulation of blood

Stimulation was carried out in sterile polypropylene tubes in a water bath at 37°C, for incubations longer than 30 minutes capped tubes were used to prevent excess evaporation. 100µL of blood was added to 100µL of stimulus. The tubes were gently rotated every 10 minutes throughout the stimulation.

6.3.6 Fixation of cells and antibody binding

At the end of the stimulation phase the tubes were placed on ice and immediately fixed with a 0.25% formaldehyde solution. (CellFIX™, Becton Dickinson). A fresh dilute formaldehyde solution was made weekly by adding 1ml CellFIX™ to 9mls water and 30mls FACSflow (Becton Dickinson). 250µL of
dilute CellFIX™ was added to each stimulation tube and left in ice for one minute. 50μL of fixed cells were added to an Eppendorf tube containing 10μL of mouse anti-human CD11b labelled with FITC (Beckman Coulter, High Wycombe, Cat IMO530) and 5μL of mouse anti-human CD16 labelled with PE for neutrophil discrimination from eosinophils (Dako, Ely, Cat R7012) or mouse anti-human CD14 labelled with PE for monocyte analysis (Dako, Ely, Cat R0864). Both antibodies were added at the same time to prevent the binding of one antibody affecting the binding of a subsequent antibody. An isotypic mouse IgG1 labelled with FITC was used to determine non specific binding. After 20 minutes on ice the samples were placed into 500μL of ice cold PBS in a polystyrene tube. 10μL of a 0.02% LDS-751 a far red fluorescent DNA dye (Cambridge Bioscience Cat No. L-7595) was added to each tube and gently agitated and placed on ice for a further 20 minutes. All samples were read on the flow cytometer within 1 hour.

6.3.7 Lysis for GAFS analysis

After 50μl of blood had been removed for assessment of CD11b levels by incubation with antibody, the remaining fixed sample was added to 1.75ml of 155mM ammonium chloride lysis buffer and agitated gently by hand and left on ice for 20 minutes. The lysed blood sample becomes visibly darker due to released haemoglobin, and was then used for flow cytometry. Samples must be analysed within 1 hour.

6.3.8 Flow cytometry

Flow cytometry was performed on a FACS Calibur machine (BD Biosciences, Cowley, Oxford). Samples were run on medium flow rate until 1000 neutrophils
were counted. Data was collected and analysed using Cell Quest v.1.2.2. (BD Biosciences, Cowley, Oxford).

For CD11b measurement gating off the red cells and platelets using the LDS-751 reading in the FL-3 channel identified the leukocyte population. Neutrophils were identified within the granulocyte cloud based on their forward and side scatter characteristics and CD16 binding, while eosinophils have are CD16 negative. Monocytes are CD14 positive. The required cell population was gated using the Cell Quest software and the geometric mean of fluorescence intensity of FITC bound to CD11b recorded.

For shape change analysis the neutrophils and eosinophils were distinguished on the basis of autofluorescence characteristics, since eosinophils have autofluorescent granule proteins. The geometric mean of forward scatter was calculated for the required cell population.
6.4 Results

6.4.1 Effect of cigarette smoke conditioned medium on levels of neutrophil CD11b

Figure 6-5 Whole blood was incubated with varying concentrations of cigarette smoke conditioned medium standardised by optical density for a period of 5-30 minutes. CD11b was measured on neutrophils. n=4 Mean ± SEM

When whole blood is incubated with CSCM there is a dose dependent increase in CD11b upregulation on neutrophils. The levels of CD11b are highest at a CSCM concentration of 2 AU, and stimulation for between 10 and 30 min gave equivalent effects. It was therefore decided to measure CD11b upregulation (neutrophils, monocytes, eosinophils) and shape change (neutrophils and eosinophils) at 10 and 20 minutes employing CSCM at a concentration of 2 AU.
6.4.2 Effect of cigarette conditioned smoke medium (CSCM) stimulation on neutrophils, eosinophils and monocytes

When whole blood was incubated with CSCM at a concentration of 2 AU for 10 minutes there was an increase in CD11b expression in all leukocyte subtypes, with an approximate doubling of levels on neutrophils. This represented an increase from a mean of 20.22 to 39.16; however this did not reach statistical significance. (p=0.08). There was an increase in CD11b on eosinophils from a mean of 36.23 to 42.91 (p=0.07), and on monocytes there was a statistically significant increase from 24.47 to 33.98 (p=0.04). As a comparator 100μM GROα was used a positive control. This caused an increase in CD11b on neutrophils from a mean of 21.81 to 77.04, n=6. Therefore at 10 minutes CSCM is not as potent a stimulus as a chemokine.
CSCM at a concentration of 2 AU caused a significant increase in CD11b upregulation on neutrophils, eosinophils and monocytes after incubation with whole blood for 20 minutes. This represented a mean increase from 17.49 to 40.25 on neutrophils (56%, p=0.026), a mean increase from 34.23 to 41.09 on eosinophils (17%, p=0.007), and an increase from 27.70 to 38.99 on monocytes. (29%, p=0.02). Therefore a stimulation time of 20 minutes was used in future experiments. The greatest increase occurs in neutrophils, and also neutrophils are more abundant in blood and hence are easier to measure using flow cytometry. For these reasons the model was developed further only studying neutrophils.

Shape change (Gated autofluorescence, GAFS): When whole blood was incubated with CSCM at a concentration of 2 AU for 10 minutes there was a non-statistically significant change in shape of neutrophils and eosinophils, representing an increase from 257.8 to 298.6 (p=0.14) in neutrophils and from 232.6 to 251.7 (p=0.10) in eosinophils. After 10 minutes incubation with a CSCM concentration of 5 AU and 10 AU it was noted that the cloud positions had shifted in a disorganised manner reflective of toxic swelling and damage, and neutrophils were showing increased autofluorescence. When whole blood was incubated with CSCM at a concentration of 2 AU for 20 minutes there was a non-statistically significant change in shape of neutrophils and eosinophils, representing an increase from 247.5 to 266.1 (p=0.09) in neutrophils and from 224.4 to 226.3 (p=0.85) in eosinophils. It was noted that the shape change was not in terms of a discrete increase in forward scatter as would be seen with a chemokine such as CXCL8, and this was taken to infer that CSCM has little chemotactic activity.
Figure 6-7 Effect of bench decay on effect of CSCM on neutrophil CD11b upregulation

Figure 6-8 CSCM was made and standardised to give a concentration of 2AU. Blood was taken from 2 subjects and incubated with CSCM for 20 minutes and neutrophil CD11b upregulation was measured to give a baseline measure of activity. The CSCM was then left at room temperature for 24 hours and the experiment repeated.

It was found that there is reduced activity of CSCM on neutrophil CD11b upregulation after CSCM has been left at room temperature for 24 hours. In subject 1 this was a change from 155% increase to 82% increase at 24 hours, while in subject 2 this was a change from 63% at baseline to -2% at 24 hours.
6.4.3 Effect of glutathione and N-acetyl cysteine (NAC) on neutrophil CD11b response to stimulation with cigarette smoke conditioned medium

Figure 6-9 Neutrophil CD11b expression was measured in whole blood after incubation with varying concentrations of CSCM for 20 minutes in the presence of 10mM Glutathione. The glutathione was added at the same time as the CSCM. n=6 Mean ± SEM. The effect of glutathione at 2AU was compared using a paired t-test. At 2AU there was no significant difference between the 2 groups, p=0.56. Glutathione is an anti-oxidant, and addition to whole blood does not reduce the stimulation of neutrophils by CSCM. It was not possible to use higher concentrations of glutathione as more concentrated solutions of glutathione caused upregulation of CD11b at baseline.
Figure 6-10 Neutrophil CD11b expression was measured in whole blood after incubation with varying concentrations of CSCM in the presence of 5mM n-acetylcysteine. The n-acetylcysteine was incubated with the CSCM for 60 minutes prior to the addition of whole blood. The blood was stimulated for 20 minutes. n=4 Mean ± SEM

N-acetylcysteine is an anti-oxidant, and after incubation for 1 hour with CSCM the stimulating effect of CSCM is not reduced. Higher concentrations of n-acetylcysteine could not be used as more concentrated solutions caused CD11b upregulation at baseline.

To demonstrate that the effect of CSCM is in some part due to an oxidant effect, incubation of CSCM was performed with superoxide dismutase and catalase to attempt to degrade active oxidants. However when this was attempted it was found that the preparations of superoxide dismutase and catalase caused CD11b upregulation on their own, possible due to preservatives in the commercially available preparations.

This is one potential limitation of this CD11b upregulation assay for the development of new drugs. It is extremely sensitive and it is possible that the
drugs under test may cause CD11b upregulation by themselves unless they are available in a pure form and are soluble in a physiological solution such as PBS.

6.4.4 Effect of removal of plasma on neutrophil CD11b response to stimulation with cigarette smoke conditioned medium.

Figure 6-6-11 Whole blood was mixed with PBS and spun at 400g to remove the plasma. The cells were re-suspended in PBS and left to rest for 60 minutes. This was then stimulated with CSCM for 20 minutes. A matched whole blood sample was stimulated simultaneously. CD11b expression on neutrophils was measured. n=6 Mean ± SEM. The groups were compared at 2AU using a paired t-test. At a CSCM concentration of 2AU there was no significant difference between the 2 groups, p=0.53. Removal of plasma from whole blood does not affect the response to stimulation with CSCM.
6.4.5 Comparison of CD11b upregulation in response to stimulation with CSCM in healthy smokers and smokers with COPD

![Graph showing CD11b upregulation](image)

Figure 6-12 Whole blood from healthy smokers and smokers with COPD was stimulated with CSCM for 20 minutes and neutrophil CD11b expression measured. n=8 MEAN ± SEM Baseline and 2AU were compared using a paired t-test. Both healthy smokers and smokers with COPD show a dose-related increase in response to stimulation with CSCM. Both groups have comparable baselines and do not differ in their neutrophil CD11b responses to stimulation with CSCM at 2AU (p=0.64).

6.4.6 Effect of menadione on CD11b upregulation on neutrophils

![Graph showing CD11b upregulation](image)

Figure 6-13 Whole blood was stimulated with menadione for times ranging from 30 to 240 minutes and CD11b measured on neutrophils. 90 minutes stimulation resulted in an increase from baseline of 9.41 to 22.68.
6.4.7 Effect of removal of plasma on neutrophil CD11b response to stimulation with menadione

Figure 6-14 Whole blood was stimulated with menadione for 90 minutes and CD11b measured on neutrophils. n=6 Mean ± SEM. The maximum response was seen at a concentration of 15µM showing an increase from baseline MFI of 12.66 to 33.05.

Figure 6-15 Whole blood was mixed with PBS and spun at 400g to remove the plasma. The cells were re-suspended in PBS. This was then stimulated with 100µM menadione for 90 minutes. A matched whole blood sample was stimulated simultaneously. CD11b expression on neutrophils was measured. n=6 Mean ± SEM. The groups were compared using a paired t-test.
There was no significant difference between whole and washed blood in the response to neutrophil CD11b upregulation in response to 100μM menadione, p=0.053.

6.4.8 Effect of glutathione on neutrophil CD11b response to stimulation with menadione.

![Graph showing the effect of glutathione on neutrophil CD11b upregulation](image)

Figure 6-16 Whole blood was stimulated with 100μM menadione for 90 minutes in the presence and absence of 10mM glutathione. n=6 Mean ±SEM. The groups were compared using a paired t-test. There was a significant reduction in neutrophil CD11b upregulation in response to stimulation with menadione in the presence of glutathione, p=0.0015.

6.4.9 Effect of xanthine/xanthine oxidase (X/XO) on neutrophil CD11b upregulation

Xanthine/xanthine oxidase causes the extracellular generation of free radicals such as superoxide. When employing whole blood with xanthine/xanthine oxidase (X/XO) I could not cause CD11b upregulation.
6.4.10 Effect of removal of plasma on neutrophil CD11b response to stimulation with xanthine/ xanthine oxidase

Figure 6-17 Whole blood was mixed with PBS and spun at 400g to remove the plasma. The cells were re-suspended in PBS. This was then stimulated with 500µM xanthine/ xanthine oxidase for 90 minutes. A matched whole blood sample was stimulated simultaneously. CD11b expression on neutrophils was measured. n=6 Mean ± SEM. The groups were compared using a paired t-test. There was a significant difference between whole and washed blood, p=0.006. There was no response to stimulation of whole blood with xanthine/ xanthine oxidase. Preliminary experiments were done on a limited number of individuals to establish stimulation time (data not shown) There was only a very small increase at 30 and 60 minutes, however at 90 minutes the increase was much greater.

Figure 6-18 Washed blood was stimulated with xanthine/ xanthine oxidase for 90 minutes and CD11b measured on neutrophils. n=5 Mean ± SEM. The maximum increase was seen at 500µM xanthine/ xanthine oxidase showing an increase from a baseline MFI of 18.22 to 36.22.
6.4.11 Effect of glutathione on neutrophil CD11b response to stimulation with xanthine/ xanthine oxidase.

![Graph showing the effect of xanthine and xanthine oxidase with and without 10mM glutathione on neutrophil CD11b upregulation.]

Figure 6-19 Whole blood was stimulated with 500μM xanthine/xanthine oxidase for 90 minutes in the presence and absence of 10mM glutathione. n=6 Mean ±SEM. The 2 groups were compared using a paired t-test. There was a significant reduction in neutrophil CD11b upregulation in response to stimulation with xanthine/xanthine oxidase in the presence of glutathione, p=0.02.

6.5 Discussion

CSCM causes a dose dependent increase in CD11b upregulation on human neutrophils which is maximal at 20 minutes stimulation. CSCM has its greatest effects on neutrophils, with much lesser effects on monocytes and eosinophils. The fact that CSCM does not have effects on neutrophil shape change at concentrations that cause large increases in CD11b upregulation may be because CSCM does not contain any chemotactic components, and this is itself interesting. The shape change that was seen with higher concentrations of CSCM may have been due to toxic apoptotic effects on the neutrophils as it was also associated with an increased auto-fluorescence of the cells. Indeed, apoptosis is a well known effect of oxidants at high doses (430).
The fact that glutathione and n-acetyl-cysteine do not have an effect on the whole blood model suggests that oxidant effect may not be the prominent effect of CSCM, or that the oxidant effect of CSCM is so great that it cannot be overcome by the concentrations of glutathione and n-acetyl cysteine used. Since CSCM had less activity in stimulating CD11b upregulation after bench decay, this suggests that the CSCM contains labile stimuli. The whole blood model required CSCM to be present at 10 times the concentration used in cell culture work (428). This may be due to the complex nature of whole blood with its proteins and oxidant buffering capacity.

CSCM caused CD11b upregulation, but I could not inhibit this activity with antioxidants, so I could not be sure that the CSCM is acting by specific oxidant mechanisms. Hence I turned to stimulation with menadione, which causes intracellular generation of ROS. Menadione caused a dose dependent increase in neutrophil CD11b upregulation, this was a greater increase in CD11b upregulation in comparison to stimulation with CSCM (3 fold versus 2 fold); and a longer time (90 min) was required to achieve CD11b upregulation. There are two possible explanations for this; menadione acts intracellularly and has to diffuse across the cell membrane before oxygen radical generation begins whereas CSCM may contain pre-formed mediators of oxidative stress. The removal of plasma does not greatly increase the effects of menadione on CD11b upregulation, this is most likely to be explained by the fact that oxygen radical generation is intracellular, and hence the plasma is not able to remove any of these stimulants before they have an effect on the cells. These results are not consistent with the results obtained by Gallin et al. (418), who found inhibition of neutrophil functions with similar concentration ranges of menadione. However, they were working with purified neutrophils in longer term...
stimulation systems and this may have affected the way in which the cells responded to the menadione.

The xanthine/xanthine oxidase system generates extracellular ROS and causes a dose dependent increase in CD11b expression on human neutrophils. For this effect to be seen plasma must be removed from the whole blood, this may be due to the presence of anti-oxidants in plasma that buffer the oxidants as soon as they are generated. Whilst having to remove plasma reduces the simplicity of a whole blood model it is still less inconvenient than having to isolate neutrophils by methods such as Percoll gradients.

I have developed menadione and xanthine/xanthine oxidase systems for stimulation of human blood. These methods could be used to screen new anti-oxidant therapies by adding therapies to the stimulation system. The observation that 10mM glutathione is able to inhibit the effect of menadione and xanthine/xanthine oxidase suggests that these stimuli are acting via a mechanism of oxidant stress. The reason that glutathione at the same concentration does not inhibit the effects of CSCM in generating CD11b upregulation may be because the CSCM is a mixture of toxic substances including oxidants, and that oxidants may not be causing the CD11b upregulation. However, the demonstration that CSCM loses activity on bench storage suggests that oxidants may still be involved.

Another system that has been used in cell culture work to generate oxidant stress is hydrogen peroxide. When hydrogen peroxide is added to blood the presence of large amounts of serum catalase leads to the rapid generation of oxygen and water and this in turn leads to effervescence of the blood. This means that this is not a suitable stimulus for our assay of whole blood CD11b.
upregulation as the physical stimulation of the cells by the effervescence leads to CD11b upregulation. Even when washed blood is used the effervescence persists due to the presence of intracellular catalase.

6.6 Conclusions

CSCM stimulation of whole blood causes a dose dependent increase in neutrophil CD11b upregulation. CSCM has little chemotactic effect on neutrophil shape change at concentrations that cause CD11b upregulation, and seems to cause toxic damage.

CSCM causes its greatest effect on neutrophils, with lesser effects on monocytes and eosinophils. The effects of CSCM could not be reversed by antioxidants (N-acetyl cysteine and glutathione). However, CSCM is less effective as a stimulant after overnight storage at room temperature, suggesting that the stimulating agent is labile and subject to decay. This would be consistent with an oxidant causing some of the effects on CD11b upregulation.

Menadione stimulation of whole blood causes a dose dependent increase in neutrophil CD11b upregulation that can be reversed by the addition of 10mM glutathione.

Xanthine/ xanthine oxidase stimulation of washed blood causes a dose dependent increase in neutrophil CD11b upregulation that can be reversed by the addition of 10mM glutathione.

Further work could demonstrate whether oxidants generated by xanthine / xanthine oxidase act on TLR2, and might prime cells for CD11b upregulation and IL-8 production when stimulating with PAMPs for TLR4 (LPS), TLR2/1, TLR2/6 (242). In addition intracellular signalling events could be studied after
menadione and X/XO, as well as the anti-inflammatory Nrf2 pathway. To establish the whole blood stimulation methods in the context of assessing drugs, we could study the effects of corticosteroids and new potent anti-oxidant agents (such as thiol anti-oxidants and Nrf2 agonists) on menadione and X/XO stimulation of CD11b upregulation. The system could also be used to define human whole blood reactions to oxidants.

6.7 Future study

The following diagram shows the signalling pathways that are activated during stimulation of extracellular receptors and that lead to the production of inflammatory mediators. It can be seen that there is a complex interaction with cross-talk of the signalling pathways. The TLR2 receptor is stimulated by oxidants and hence forms the basis of further study of the inflammation generated as a result of oxidant stress.

![Diagram of signal transduction pathways during cellular activation](image)

Figure 6-20 Signal transduction pathways during cellular activation. Based on (416) (242)
6.7.1 TLR2 involvement

We speculate that extracellular oxidants (CSCM and X/O) may cause neutrophil activation via TLR2 (Toll-like receptor, TLR-2), as has been recently claimed by other workers when studying a CSCM stimulation system with human blood (242). TLR2 is a cell surface "pattern recognition receptor" (PRR) for "pathogen-associated molecular patterns" (PAMPs), that may recognise oxidants. One could use specific MoAbs against TLR2 to see if one can block the effects of extracellular oxidants on CD11b upregulation. These effects could be compared in neutrophils and monocytes, and we need to compare menadione and X/O stimulation in neutrophils and monocytes. Flow cytometry can also be used to detect levels TLRs on the cell surface in response to stimulation. (431)

6.7.2 Priming by extracellular oxidants: TLR4 agonists

We speculate whether X/O may cause priming for immune surveillance to Gram negative and Gram positive bacteria, as has been recently demonstrated with CSCM and human blood cells (242). Further work could assess if oxidants generated by X/O might prime cells for CD11b upregulation and IL-8 production when stimulating with PAMPs (agonists) for TLR4 (LPS), TLR2/1 (Pam3CSK4), and TLR2/6 (FSL-1) (242). This would involve studying CD11b upregulation in the presence of these separate agonists, and then looking for priming effects of extracellular oxidants. Oxidant stress activates p38 MAP kinase in an Src-dependent manner and oxidants alter the response to LPS induced activation p38 MAP kinase in macrophages (234). Cigarette smoke induced IL-8 production by monocytes can be blocked by a TLR-4 inhibitor and an inhibitor of NF-κB (241).
6.7.3 Cell signalling

It would be interesting to study rapidly activated intracellular signalling after stimulation with CSCM, menadione and X/XO. There is generally considerable cross-talk involved in activation by oxidants via TLR2. TLR2 activation may initially involve IRAK, and then MAPk/AP-1, NF-κB and IRF (IFN regulatory factors). Reactive oxygen species (ROS) may also deplete intracellular glutathione, and initiate the anti-inflammatory Nrf2 pathway. Nrf2 activation acts on ARE (antioxidant response element), and Nrf2 agonists have the potential to counteract the effects of oxidants (Kensler TW et al. 2006).

6.7.4 Development of a preclinical screening system for new drugs

To establish the whole blood stimulation methods in the context of assessing drugs, we could study the effects of corticosteroids and new potent anti-oxidant agents (such as thiol anti-oxidants and Nrf2 agonists) on menadione and X/XO stimulation of CD11b upregulation. It would also be interesting to see the effects of inhibitors of intracellular signalling on CD11b upregulation, eg. MAP kinase inhibitors and IKK2 inhibitors. Finally we could develop automated additional readouts: mRNA for induction of proteins (e.g. IL-8), measurement of generation of IL-8 into supernatant after some hours of stimulation. These in vitro systems could also be employed ex vivo in subjects after taking novel anti-oxidant drugs.

6.7.5 Assessment of primed status in patients

We could develop human whole blood systems for monitoring inflammation/oxidant status in patients. These could measure CD11b basal levels, and then perform dose responses for stimulation with X/XO and menadione in terms of CD11b, additionally looking at maximal CD11b levels. It
would be interesting to study this system in subjects after smoking, and in situations such as COPD smokers compared with healthy smokers.

7 Discussion and Conclusions

7.1 Discussion

The nasal allergen challenge model has great potential as an *in vivo* model of allergic inflammation; since it involves mast cell degranulation, and recruitment of Th2 cells and eosinophils. Studies presented in this thesis show it to be safe and well tolerated by subjects. By defining those mRNA species and proteins, as well as inflammatory mediators and transcription factors, it allows us to define targets for asthma and allergy therapy. It also allows the collection of multiple samples following a single challenge to demonstrate the pharmacodynamics of a drug. The model is also well suited to cross-over design trials allowing the effects of a novel therapeutic agent to be studied as a dose response and compared to placebo.

A variety of samples can be collected from the nose. Nasal lavage samples can be collected to determine eosinophil levels, and nasal secretions can be collected by a SAM to allow for cytokine and chemokine analysis. A small scrape of the nasal epithelial mucosa can also be collected to allow for mRNA and genomic studies.

Studies presented in this thesis show very disappointing results for filter paper eluates using the Whatman's 42 filter paper, despite this being the paper used in the original description of the method (258), and also on the CSU by Erin and colleagues (50;100;101) Analysis of data for the single allergen challenge repeatability and dose response failed to show increased levels of IL-4, IL-5 and
Il-13, and the expected late response was not found. Because of a delay in availability of the correctly processed data for this study, we had already embarked on 2 further studies with the same deficient method: the repeat dose NAC and the nasal cigarette smoke challenge which were conducted in parallel. The lack of response in the subsequent two trials lead to a review of the data for all 3 studies: this showed that single dose NAC did not cause a consistent late phase response for IL-4, IL-5 and IL-13.

Faced with these negative results, I was involved in a detailed review of the NAC methodology, and helped make a series of recommendations on how the NAC could be modified to be able to detect levels of IL-4, IL-5 and IL-13 in the late phase. I contributed to the design of a NAC study that was carried out in the first quarter of 2007, but did not carry out the clinical or laboratory work. This study employed novel synthetic absorptive media (SAM) instead of Whatman's filter paper No. 42. SAM is a synthetic fibrous hydroxylated polyester which has been specifically developed to have low protein binding, whereas Whatman's filter paper is derived from cotton plant and is subject to an acid wash during its manufacture and is not subject to quality control with regards to its protein binding activity. In addition, a higher dose of Phleum pratense allergen was employed. This study was very successful and IL-4, IL-5 and IL-13 levels were consistently over 100pg/ml in the majority of individuals in the late phase: these high levels have never previously been reported, nor found in our laboratory. Hence, we think that we have potentially solved the technical problems I encountered in my thesis.

However, on a positive note, data presented in this thesis suggests that a repeat dose model of NAC causes increased levels of cytokines in nasal lavage
samples with repeated allergen, and hence repeat NAC may be useful for testing certain new drugs, and higher initial levels of cytokines increases the power to detect changes from a novel therapeutic agent. Repeat NAC is especially useful when looking at a potentially immuno modulatory agent, that may operate on altering T cell differentiation in the presence of allergenic stimulation.

New anti-inflammatory therapies are also needed in the treatment of COPD, and a non-invasive model would be useful to assess new drugs early in clinical development, although this might need to relate to oxidants, neutrophils and macrophages, as opposed to mast cells, Th2 cells and eosinophils. Patients with COPD are generally over 40 years of age and have other co-morbidities as a result of their smoking and hence a model that removes the need for them to have invasive procedures such as bronchoscopy is desirable. The nasal cigarette smoke challenge shows potential as a model although a number of modifications to enhance its usefulness are required. In particular we need to assess this method with the new SAM, rather than Whatman’s No 42 filter paper.

It has previously been shown that correcting for the weight of secretions obtained using the filter paper method does not effect the results in terms of levels of cytokines measured (95). On review of the filter paper weights obtained during the studies presented in this thesis it has been shown that there is an increase in weight of secretions during the early phase (5 min to 30min) following allergen challenge, compared to prechallenge nasal secretion weights. However the weights obtained during the late phase following allergen challenge (2 to 8h post challenge) are not significantly different from those
obtained prior to challenge, and remain remarkably constant with a weight of 34.36 (19.82-48.91) μg (mean, 95% confidence interval). Hence correcting for weight does not change the overall level of cytokines when studying the late phase response. The aim of the studies presented here was to develop a model of allergen challenge to measure the Th2 cytokines IL-4, -5 and -13 during the late phase, and such a model does not require correction for weight of secretions. In the cigarette smoke nasal study the weight of secretions obtained did not vary significantly across the whole study period with a weight of 39.89 (27.05-52.73) μg (mean, 95% confidence interval) and hence correcting for weight of secretions does not affect the overall comparison of cytokine levels.

Whole blood stimulation in vitro is an ideal technique for initial screening of some anti-inflammatory compounds. It does not require subjects to be exposed to new drugs, yet human cells are stimulated which has distinct advantages over the use of animal models especially when studying the immune system which may have important differences between the species. In addition subjects could take a novel therapeutic agent and then their blood taken at intervals and stimulated ex vivo. Blood sampling allows for the pharmacokinetics of a drug to be determined but importantly this stimulation of whole blood model could allow for the associated pharmacodynamics of the drug to be determined. Development of this model to use allergen, LPS or other stimulators of TLRs will increase the scope of usefulness of this model. The model presented in this thesis would allow for the screening of new anti-oxidant compounds for the treatment of COPD. A drawback with this methodology is that many drugs do not act on blood cells, and effects on blood may differ compared to tissue.
effects. Nevertheless, for drugs such as chemokine receptor antagonists, this methodology holds great promise.

Non-invasive biomarkers that are currently in use for the assessment of respiratory disease have a series of technical drawbacks. Exhaled breath condensate has the problem of contamination, there is a high chance that samples become contaminated by saliva and the presence of cells from the buccal mucosa and even airborne keratin flakes makes genomic analysis difficult to interpret (432). Sputum is often studied as it is relatively easy to obtain and can reflect inflammatory changes in the lower airways. However the only real proven clinical usefulness of sputum is the determination of eosinophil counts in asthma. The difficulties with determining the significance of finding increased levels of inflammatory mediators in sputum is that it is not possible to determine how long the sample has been sitting in the bronchial tree and hence its relationship to the timing of a challenge. It is also often contaminated by bacteria which may effects levels of proteins, and also it is possible that inflammatory cells may have undergone lysis and released their intracellular stores of chemokines and cytokines. In addition the release of a fluid phase for determination of cytokine and chemokine levels has numerous technical difficulties. The use of ultrasonication may cause cell lysis and cause falsely elevated protein levels, and treatment with the reducing agent dithiothreitol (DTT) may lead to a reduction in the levels of proteins measured (433). The SAM described in this thesis to obtain nasal secretions could be utilised down the sampling channel of a bronchoscope to obtain epithelial lining fluid, this would remove all the problems associated with sputum analysis and would allow for correlations between changes in the upper and lower airways.
Any new biomarkers that are developed must be extensively validated and their repeatability and usefulness in clinical practice determined. A relationship between the biomarker and the disease state must be established and levels of the biomarker must correlate with disease severity and/or progression. A reduction of biomarker levels due to a novel therapeutic agent must be shown to translate to reduction in disease severity. Examples where biomarkers have been useful include glucose levels in diabetes, cholesterol levels in cardiovascular disease, and viral load in HIV infection. The use of a challenge situation to increase the level of a biomarker must reproduce the changes that occur in the disease state. The advantage of a challenge model is that it allows for the collection of serial samples for biomarker analysis following challenge which permits assessment of pharmacodynamic properties.

Biomarkers can be used during early phase studies as a guide to dose selection and escalation, and can also be used to rapidly screen new drugs to eliminate drugs before they progress to costly and complex large clinical trials. This model of drug development does have limitations in that biomarkers do not identify potential side-effects and may cause misleading results. An example of this is that the drug milrinone appeared to cause haemodynamic changes that would be beneficial in heart failure; however in a longer term clinical trial it increased mortality patients with heart failure (434).

Once a biomarker has been validated for a particular disease it could be used in the diagnosis and monitoring of the disease. It could also be used to guide the choice of therapeutic agent and also to monitor the response to any new therapies.
The future of determining pathogenesis of disease and hence drug development lies in the exploration of the genetics of disease in relation to environmental and infectious influences. Rapid analysis of gene expression (mRNA) is now possible with such techniques as Affymetrix. These mRNA microarrays allow screening of thousands of genes for differences in expression or differences in how genes are connected in molecular networks (435). It is possible to study genes (SNPs) and gene expression in normal and disease-associated tissue, and it has been proposed that it would have clinical value in the characterisation of interstitial lung disease (436). The use of gene expression profiling in asthma has also been studied. It has been shown that airway epithelial cells in asthma have a distinct activation profile and changes within the gene expression can identify differences in clinical responsiveness to treatment with corticosteroids (437). This an extremely important advance as it show the value of pharmacogenetics whereby therapies are chosen based on an individual patients genetic profile to ensure that only the most effective treatments are given. An example of this that is already in widespread clinical use in the use of trastuzumab in patients whose tumour cells show evidence of HER2 protein expression (438).

Knowledge of the mechanisms of disease can also help to identify gene target. A study of normal and asthmatic bronchial biopsies has shown that IL-4 and IL-13 regulate the squamous cell carcinoma antigens (SCCA) and that serum levels of SCCA were raised in the serum of asthmatic suggesting that SCCA may be an important biomarker in asthma (439). DNA microarray can also examine the expression of genes that occur over time following a challenge. Bronchial epithelial cells show three distinct phases of gene expression following challenge with oxidants (440) showing the complex nature of disease.
pathogenesis and the importance of the need to understand the changes that occur at various time point following a challenge. The interaction of genes has lead to the suggestion that this should be viewed as a systems genetics model of disease that takes into account this complexity (441).

This thesis has shown that biomarkers can have usefulness in the study of allergic inflammation. The use of a SAM can considerably improve the detectable levels of cytokines form nasal secretions. Whole blood has the potential to be used to screen new anti-oxidants for the treatment of COPD and possibly other diseases where oxidant stress is a significant factor in the aetiology. However biomarkers do have their limitations and they must be carefully validated and their relevance to the clinical situation established.

7.2 Conclusions

NAC is a safe effective model for screening new drugs; however nasal secretions must be collected using a SAM that has been validated for use with human proteins.

A model of repeated doses of nasal allergen may lead to an increase in levels of IL-4, IL-5 and IL-13 in nasal secretion.

Cigarette smoke nasal challenge has the potential to be used to characterise the inflammatory changes that occur in smoking related lung disease and also to be used for the evaluation of new anti-inflammatory therapies for the treatment of COPD

CSCM stimulation of whole blood causes a dose dependent increase in neutrophil CD11b upregulation. CSCM causes its greatest effect on neutrophils,
with lesser effects on monocytes and eosinophils. The effects of CSCM could not be reversed by anti-oxidants (N-acetyl cysteine and glutathione).

Menadione and xanthine/ xanthine oxidase stimulation of whole blood causes a dose dependent increase in neutrophil CD11b upregulation that can be reversed by the addition of 10mM glutathione.
8 Future work

8.1 Nasal allergen challenge

1. One should only employ Whatman's filter paper if it has appropriate elution characteristics for a particular batch demonstrated by spiking with commercial standards and nasal samples. Given the complexities of such an approach, there is a need to identify and validate novel absorptive matrices with reproducible and efficient elution characteristics.

2. Determination of maximum dose of NAC and further determination of dose response effects of NAC.

3. It would be beneficial to determine subjects' responsiveness to allergen challenge at screening prior to inclusion in the study, with documentation of serial nasal cytokine and chemokine responses.

4. Use of nasal scrape samples to assess events at a tissue level including proteomic analysis. In addition nasal cells could be cultured to determine in vitro responses to cytokine challenge.

8.2 Nasal cigarette smoke challenge

1. Characterise subjects further at screening with CT scans and full lung function, to ensure that "healthy" group are free of any respiratory disease.

2. Collect samples at time points beyond 8 hours.

3. Refine laboratory techniques of nasal lavage cytology and also determine the elution characteristics of an alternative matrix for the collection of nasal filter paper samples.
8.3 Blood oxidant challenge model

1. Determine extent of TLR2 involvement in oxidant signalling

2. Study effects of priming by extracellular oxidants using TLR4 agonists

3. Study cell signalling pathways involved in CD11b upregulation in relation to oxidant stimulation

Helen Neighbour, Grant C. Nicholson, Louise E. Donnelly, Peter J. Barnes, Irfan Rahman, Onn Min Kon, Trevor T. Hansel Effects of oxidant-menadione on human blood neutrophil CD11b levels assessed by flow cytometryThematic Poster ERS 2005
10 Acknowledgements

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I would like to thank Grant Nicholson for teaching me the flow cytometry protocol and also for his general advice around laboratory practise and for checking all my dilution calculations. I am grateful to Peter Fenwick and Louise Donnelly for their help with the generation of CSCM and discussions on the use of CSCM. Professor Ian Adcock and Professor Irfan Rahman gave helpful advice on the use of oxidant stimuli. Zarin Brown and Jennifer Willis of Novartis helped with the measurement of cytokines in nasal lavage in the repeated nasal allergen study and cigarette smoke nasal challenge. I am grateful to Richard Florio of the Histopathology department of the Royal Brompton Hospital for his help with interpreting the nasal cytology slides from the cigarette smoke nasal challenge study.
I would like to thank Tom Conway for his help with all the paperwork and to Andrew Tan for help with Ethics submissions and also during the clinical phase of the studies. Huge thanks must go to Linda Green for all her help with subject recruitment and obtaining the clinical samples. Also her friendship and advice during my time at the Clinical Studies Unit and subsequently has proved invaluable in seeing this Thesis completed.

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Finally I would like to wish Trevor and the Clinical Studies Unit all the best for the future. I hope that I can continue to use the skills, knowledge and principles for the conduct of great clinical trials in my future career.
11 Appendices

11.1 Appendix 1 Nasal Symptom Score Card for Nasal Challenge

Please complete the following table on hay fever symptoms, entering numbers between 0 to 3

0 – Absent symptoms (no sign/symptoms evident)

1 – Mild symptoms (sign/symptoms clearly present, but minimal awareness; easily tolerated)

2 – Moderate symptoms (definite awareness of sign/symptom that is bothersome)

3 – Severe symptoms (sign/symptom that is like severe hay fever).

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<thead>
<tr>
<th>Subject initials:</th>
<th>Subject code:</th>
<th>randomisation</th>
<th>Date:</th>
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<td>0 15 min 30 min 1hr 2hr 3h 4h 5h 6h 7h 8h</td>
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<td>Nasal Obstruction: Have you had difficulty in breathing through a blocked nose today or since your last assessment today?</td>
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<td>Rhinorrhoea: Has your nose been running today or since the last assessment today?</td>
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<td>Nasal Itch: Has your nose been itching today or since the last assessment today?</td>
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<td>Sneezing: Have you been sneezing today or since the last assessment today?</td>
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<td>Total Nasal Symptom Score (TNSS)</td>
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11.2 Appendix 2 Whatman's filter paper (Number 42) versus Synthetic Absorptive Matrix (SAM) (Accuwik Ultra, Pall Gellman) for sampling nasal secretions

Failure to detect significant levels of IL-4, IL-5 and IL-13 in nasal filter paper eluates prompted a comparative analysis of potential reasons (see discussion in Chapter 3). A potential reason was considered to be batch variability in Whatman's filter paper, and for this reason a series of experiments were carried out to compare the Whatman's filter paper with SAM (Accuwik Ultra). I contributed to the design of these experiments that were carried out by Mr Grant Nicholson.

11.2.1 In vitro spiking experiment: Comparison of Accuwik and Whatman paper with Beadlyte standard

Whatman's filter paper no. 42 is designed for filtration, and limits to protein-binding are not included in the specification. Cellulose is a natural product from the cotton plant, and there may therefore be variation in protein-binding potential between batches, especially since there is a final step involving washing the paper in acid. (Conversations with Mr. Giles Barton, Head of Technical Department, Whatman International Ltd. Tel: 01622 676670).

Conversely, Accuwik Ultra SAM (manufactured by Pall Corporation) is fibrous hydroxylated polyester, with low protein binding, and free of binders and surfactants. Each batch has quality assurance for protein-binding, and this medium is used during biological immuno-assays (holding monoclonal antibody reagents) and for sampling of urine in pregnancy-testing kits.

Beadlyte pooled standard contains known levels of 26 cytokines and chemokines and was applied to Accuwik and Whatman's paper. 100 microlitres
of Beadlyte pooled standard was added to two of the paper strips (30x7mm). The strips were placed in an Eppendorf containing Beadlyte assay buffer (500ul). The Eppendorf were briefly vortexed and placed on a rolling mixer for 10 minutes. The tubes were then centrifuged at 400g for 10 minutes, following which the supematant was removed, vortexed and analysed by Luminex.
11.2.2 Ex vivo NAC on a single hay fever sufferer out of season:
Comparison of detectable levels of IL-4, IL-5 and IL-13 using Whatman's versus Accuwik SAM

The results clearly show enhanced chemokine/cytokine recovery using the Accuwik paper for IL-4, IL-5 and IL-13.

On 1st September 2006, one subject (TTH) with confirmed allergic rhinitis to Timothy grass pollen, was given NAC with timothy grass pollen (500BU each nostril) from the Dolphin delivery device. The NAC was preceded by nasal lavage with PBS in each nostril (20 times over 1 minute). Sampling was undertaken by placing Accuwik in the left nostril and Whatman's in the right nostril (2 papers/nostril), and leaving in situ for one minute at the time points up to 6 hours.

The results indicate clearly that Accuwik paper is superior to Whatman 42 in absorbing nasal fluid and releasing higher detectable levels of IL-4, IL-5 and IL-13.
To ensure reliable results in future, the NAC technique will be simplified and enhanced by using Accuwik paper, and diluting straight into commercially available Luminex assay buffer. Accuwik ultra paper is synthetic and has quality control for protein elution and individual batches should be tested for elution of a standard source of IL-4, IL-5, IL-13 (commercial standard and biological positive control).

11.3 Use of SAM in a clinical trial of a novel therapeutic agent using the nasal allergen challenge model

Results are shown for a clinical trial carried out on the NHLI CSU that employed a single oral dose of a novel therapeutic given 30 min before a NAC. There were major changes employed in this study, compared with a previous published study with nasal filter paper (100):

Dose of timothy grass pollen increased from 500 BU (0.3µg of Phleum pratense allergen per nostril) to 1.0 µg of Phleum pratense allergen per nostril

Accuwik Ultra SAM was employed instead of Whatman’s filter paper number 42.

Data is shown for levels of IL-4, IL-5 and IL-13 in individual subjects, and it should be noted that in 8 of 12 subjects there are demonstrable significant late phase increases in IL-4, IL-5 and IL-13 to levels ≥100pg/ml.
Figure 11-1 Levels of IL-4 detectable in Accuwik Ultra eluate in relation to nasal allergen challenge
Figure 11-2 Levels of IL-5 detectable in Accuwik Ultra eluate in relation to nasal allergen challenge
Figure 11-3 Levels of IL-13 detectable in Accuwik Ultra eluate in relation to nasal allergen challenge
Figure 11-4 Effect of a novel therapeutic agent on cytokine levels in nasal SAM eluate following NAC. Subjects receiving active drug are shown in red. There is a statistically significant effect of the drug on IL-13 levels at 8 hours post challenge.

In this thesis Whatman's filter paper was employed in:

- NAC repeatability and dose response (Chapter 3)
- Repeated NAC on 4 consecutive days (Chapter 4)
- Cigarette smoke nasal challenge (Chapter 5)
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Ref Type: Generic


Ref Type: Generic


Ref Type: Generic

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